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| (54) Title: METHODS AND REAGENTS FOR HLA DRBETA DNA TYPING | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| <p>DRB1 Type Patterns</p> <table border="1"><thead><tr><th>Pattern</th><th>Primer</th></tr></thead><tbody><tr><td>DRB1*010102</td><td>○ □</td></tr><tr><td>DRB1*0103</td><td>○ □</td></tr><tr><td>DRB1*1518</td><td>□ □</td></tr><tr><td>DRB1*0201</td><td>□ □</td></tr><tr><td>DRB1*0202</td><td>□ □</td></tr><tr><td>DRB1*0301</td><td>□ □</td></tr><tr><td>DRB1*0302</td><td>□ □</td></tr><tr><td>DRB1*0401</td><td>□ □</td></tr><tr><td>DRB1*0402</td><td>□ □</td></tr><tr><td>DRB1*040304050407</td><td>□ □</td></tr><tr><td>DRB1*04040408</td><td>□ □</td></tr><tr><td>DRB1*04050410</td><td>□ □</td></tr><tr><td>DRB1*0409</td><td>□ □</td></tr></tbody></table> | | | | Pattern | Primer | DRB1*010102 | ○ □ | DRB1*0103 | ○ □ | DRB1*1518 | □ | DRB1*0201 | □ | DRB1*0202 | □ | DRB1*0301 | □ | DRB1*0302 | □ | DRB1*0401 | □ | DRB1*0402 | □ | DRB1*040304050407 | □ | DRB1*04040408 | □ | DRB1*04050410 | □ | DRB1*0409 | □ |
| Pattern | Primer | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| DRB1*010102 | ○ □ | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| DRB1*0103 | ○ □ | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| DRB1*1518 | □ | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| DRB1*0201 | □ | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| DRB1*0202 | □ | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| DRB1*0301 | □ | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| DRB1*0302 | □ | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| DRB1*0401 | □ | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| DRB1*0402 | □ | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| DRB1*040304050407 | □ | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| DRB1*04040408 | □ | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| DRB1*04050410 | □ | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| DRB1*0409 | □ | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| (57) Abstract | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| <p>Primers for amplification of specific nucleic acid sequences of the second exon of HLA DRbeta genes and probes for identifying polymorphic sequences contained in the amplified DNA can be used in processes for typing homozygous or heterozygous samples from a variety of sources and for detecting allelic variants not distinguishable by serological methods. This HLA DRbeta DNA typing system can be used in a dotblot format that is simple and rapid to perform, produces detectable signals in minutes, and can be used for tissue typing, determining individual identity, and identifying disease susceptible individuals.</p> | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |

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Methods and Reagents for HLA DRbeta DNA Typing

Cross-Reference to Related Applications

This application is a continuation-in-part of copending Serial No. 623,098, filed December 6, 1990, which is a continuation-in-part of copending Serial No. 491,210, 5 filed August 15, 1989, which is a continuation of now abandoned Serial No. 899,344, filed August 22, 1986, which is a continuation-in-part of now abandoned Serial No. 839,331, filed March 13, 1986, each of which is incorporated herein by reference.

Background of the Invention

Field of the Invention

10 The present invention provides methods and reagents for DNA typing HLA DRbeta (DRB) nucleic acids. The invention enables one to type homozygous or heterozygous samples from a variety of sources, including samples comprising RNA or cDNA templates, and to detect allelic variants not distinguishable by present serological, cellular, or biochemical methods. The present typing system facilitates 15 typing tissue for transplantation, determining individual identity, and identifying disease susceptible individuals. The invention therefore has applications in the field of medicine generally and medical research and diagnostics specifically, the field of forensic science, and the field of molecular biology.

Description of Related Art

20 The HLA Class II proteins HLA DR, HLA DQ, and HLA DP are encoded by genes in the major histocompatibility complex (MHC) region on the short arm of human chromosome 6. The Class II proteins are heterodimeric glycoproteins consisting of an approximately 34 kD alpha chain and an approximately 29 kD beta chain. The Class II proteins are expressed on the cell surface of macrophages, B-cells, 25 activated T-cells, and other cell types and are involved in binding and presenting antigen to helper T-lymphocytes. See the article entitled "Structure, function, and genetics of the Human Class II molecules" by Giles and Capra, 1985, *Adv. Immunol.* 37:1. In addition, the Class II proteins influence specific immune responsiveness by determining the repertoire of expressed T-cell receptors in mature T-lymphocytes. For 30 a general review of the HLA Class II genes and proteins, see the article entitled "Structure, sequence and polymorphism in the HLA D region" by Trowsdale *et al.*, 1985, *Immunol. Rev.* 85:5.

The Class II alpha and beta chains are encoded by separate genes, and the DP, DQ, and, DR genes are located in separate regions of the MHC. In the DR region, a 35 single DRA locus, or gene, encodes the non-polymorphic DRalpha chain, but five

different DRB loci, termed DRB1, DRB2 (now known as DRB6), DRB3, DRB4, and DRB5, encode the polymorphic DRbeta chain. Some loci are present only on certain haplotypes (such as DRB5 on the DR2 haplotype); in addition, the number of expressed DRB genes also varies between haplotypes. See the article entitled "HLA-

- 5 DRbeta genes vary in number between different DR-specificities, whereas the number of DQbeta genes is constant" by Bohme *et al.*, 1985, *J. Immunol.* 135:2149.

The number of distinct DRB1 alleles identified is continually increasing. The 1989 report from the WHO Nomenclature Committee for factors of the HLA system identified 34 distinct DRB1 alleles; these alleles are thought to express the serological 10 DR specificities DR1 to DRw18. (see the article entitled "Nomenclature for factors of the HLA system, 1989" by the WHO Nomenclature Committee, 1990, *Immunogenetics* 31:131-140, incorporated herein by reference). By the 1990 report, the number of DRB1 alleles recognized had risen to 45 (see the article entitled "Nomenclature for factors of the HLA system, 1990" by the WHO Nomenclature 15 Committee, 1991, *Immunogenetics* 33:301-309, incorporated herein by reference). The present invention provides the sequences of several newly discovered alleles.

The alleles of the DRB2 locus (now termed the DRB6 locus), which are present on DR1, DR2, and DRw10 haplotypes, are apparently not expressed. See the article entitled "Analysis of isotypic and allotypic sequence variation in the HLA DRB region 20 using the *in vitro* enzymatic amplification of specific DNA segments" by Erlich *et al.*, 1989, in *Immunobiology of HLA* (Dupont ed., Springer-Verlag, New York).

The alleles of the DRB3 locus, which is thought to encode the supertypic specificity DRw52 (DRw52a, DRw52b, and DRw52c), are present on the DR3, DRw6, DRw11, DRw12, DRw13, DRw14, DRw17, and DRw18 haplotypes.

25 The DRB4 locus, which has a single allele, encodes the DRw53 supertypic specificity and is present only on the DR4, DR7, and DRw9 haplotypes. See the article entitled "Structural relationships between the DRbeta1 and DRbeta2 subunits in DR4, 7, and w9 haplotypes and the DRw53 (MT3) specificity" by Matsuyama *et al.*, 1986, *J. Immunol.* 137:934.

30 The alleles of the DRB5 locus are present only on DR2 haplotypes. See the article entitled "Analysis of isotypic and allotypic sequence variation in the HLA DRB region using the *in vitro* enzymatic amplification of specific DNA segments," *supra*.

35 Polymorphism of the Class II DR antigens (proteins) is currently typed with allosera obtained from multiparous women in a microcytotoxicity assay on purified B lymphocytes. In addition, cellular typing protocols capable of greater specificity and based on either the specificity of alloreactive T-cell clones or the proliferative response of T-cell cultures to stimulation by homozygous typing cells (HTCs) have been developed.

These cellular-based analyses define the Dw specificities that further subdivide many of the serologically defined antigens, e.g., the five Dw subtypes of DR4. See the article entitled "Sequence polymorphism of HLA DRbeta I alleles relating to T cell recognized determinants" by Cairns et al., 1985, *Nature* 317:166. Both the serological and cellular assay procedures, however, are difficult and time-consuming. HLA DR DNA typing protocols based on restriction fragment length polymorphisms (RFLP) have also been developed. See U.S. Patent No. 4,582,788, incorporated herein by reference. However, these RFLP-based analyses require large amounts of high molecular weight DNA, are labor intensive, and the limited number of informative restriction enzymes in turn limits the results obtained.

The advent of the polymerase chain reaction (PCR) has facilitated the analysis and manipulation of complex genomic DNA. The PCR process enables one to amplify a specific sequence of nucleic acid starting from a very complex mixture of nucleic acids and is more fully described in U.S. Patent Nos. 4,683,195; 4,683,202; 15 4,889,818; and 4,965,188, and European Patent Publication Nos. 237,362 and 258,017, each of which is incorporated herein by reference.

The PCR process has also facilitated typing the Class II HLA DNA of an individual. Scientists have studied the polymorphic second exon of DRB loci in genomic DNA by designing oligonucleotide primers and using those primers to amplify the sequences of interest. See the article entitled "Sequence analysis of the HLA DRB and HLA DQB loci from three *Pemphigus vulgaris* patients" by Scharf et al., 1988, *Hum. Immunol.* 22:61.

When the PCR primers contain restriction enzyme recognition sequences, the amplified DNA can be cloned directly into sequencing vectors, and the nucleotide sequence of the amplification product can be readily determined. See the article entitled "Direct cloning and sequence analysis of enzymatically amplified genomic sequences" by Scharf et al., 1986, *Hum. Immunol.* 23:1076.

The amplified DNA can also be studied by detection methods that employ sequence-specific oligonucleotide (SSO) probes. See the article entitled "Analysis of enzymatically amplified beta-globin and HLA DQalpha DNA with allele-specific oligonucleotide probes" by Saiki et al., 1986, *Nature* 324:163.

Despite these advances, the complexity of the HLA DRbeta genes has prevented the development of an informative and efficient means for determining the HLA DRbeta DNA type of an individual. The present invention meets the need for an efficient, informative DRbeta DNA typing method by providing novel processes and reagents. These novel processes and reagents have in turn led to the discovery of previously unknown DRbeta alleles, which can also be typed and identified by the present method.

The present typing system can be used to type cDNA synthesized from mRNA and to type and study the expression of DRB genes in tissues, transgenic systems, disease states, and cells lines. Cells that do not express the DR antigens or show unusual seroreactivity, such as tumor cells, can be readily typed. Moreover, samples 5 from unusual sources, e.g., ancient DNAs or forensic samples, can be typed, even when the DNA sample is degraded or when only very small quantities are available for analysis.

Because PCR can amplify a fragment of target DNA over a million-fold, and because the present system can employ PCR-generated nucleic acid, radioactively 10 labeled probes are not necessary, and nonisotopic SSOs covalently coupled to horseradish peroxidase (HRP) provide sufficient sensitivity for detection. The presence of the specifically bound HRP-labeled probes of the invention can be detected in a simple dot-blot format by chromogenic dye or chemiluminescent substrates in a matter of minutes.

15 Summary of the Invention

The present invention provides amplification and detection methods, generic and allele or group specific amplification primers, nonisotopic sequence specific oligonucleotide (SSO) probes, including probes for identifying previously unknown alleles at the DRB1 locus, and kits for practicing the methods, that together provide a 20 rapid, simple and precise system for typing the HLA DRB alleles, including those that cannot be distinguished by serological methods.

In one aspect, the present invention provides a method for determining the DRbeta DNA type of nucleic acid in a sample, which method comprises (a) amplifying any nucleic acids in the sample that contain a DRbeta gene second exon; (b) hybridizing 25 said nucleic acid amplified in step (a) to a first panel of oligonucleotide probes under conditions such that said probes hybridize only to exactly complementary sequences greater than ten nucleotides in length; (c) amplifying a specific subset of nucleic acids in the sample that contain DRbeta gene second exon sequences; (d) hybridizing said nucleic acid amplified in step (c) with a second panel of oligonucleotide probes under 30 conditions such that said probes hybridize only to exactly complementary sequences greater than ten nucleotides in length; and (e) determining from the pattern of probe hybridization in steps (b) and (d) the DRbeta alleles from which the DRbeta DNA in said sample originates.

In another aspect, the present invention provides a method for determining 35 whether a sample comprises nucleic acid from the DRB1 allele of a serological type selected from the group consisting of the DR1, DR2, DR4, DR7, DRw9, and DRw10 types, which method comprises (a) amplifying the DRB1 nucleic acid in the sample;

and (b) hybridizing said nucleic acid amplified in step (a) to a panel of oligonucleotide probes under conditions such that said probes hybridize only to exactly complementary sequences greater than ten nucleotides in length, and wherein said probes hybridize to unique polymorphic sequences encoding amino acid residues 9 to 16 of the DRB1
5 second exon.

Brief Description of the Figures

Figure 1 shows the results of DRB generic and DRB1 specific amplification and detection; see Example 2.

10 Figure 2 shows the results of DRB1 specific amplification and detection; see Example 2.

Figure 3 shows the results of HLA DRB DNA typing of the classical serological types DR1 through DR10; see Example 3.

Figure 4 shows the results of HLA DRB DNA typing to subtype cell lines with the DR4 serological specificity; see Example 5.

15 Figure 5 shows the results of HLA DRB DNA typing to subtype the DR3, DR5, DRw6, and DRw8 specificities; see Example 6.

Figure 6 shows the tabulation of probe hybridization results to determine DRB type; see Example 6.

20 Figure 7 shows the results of HLA DRB DNA typing of a number of different cell lines; see Examples 6 and 7.

Figure 8 shows the HLA DRB DNA subtyping of DR3 cell lines; see Example 7.

Figure 9 shows the tabulation of probe hybridization results to determine the HLA DRB DNA type of heterozygous and other unusual samples; see Example 7.

25 Figure 10 shows the tabulation of probe hybridization results to determine the HLA DRB DNA type; see Example 9.

Figure 11, 12, and 13 show the tabulation of probe hybridization results to determine DRB allele type; see Example 9.

Detailed Description of the Invention

30 The present invention provides an HLA DRB typing system and sequence specific oligonucleotide probes (SSOs) for analyzing DRB alleles. The invention can be used to type heterozygous samples from a variety of sources, including cDNA templates, and can be used to detect allelic variants not distinguishable by serological methods. This typing system can utilize a dot-blot format that is simple and rapid to perform, produces detectable signals in minutes, and will prove valuable for tissue typing and determining individual identity and disease susceptibility.

The present invention provide methods for detecting and identifying HLA DRB alleles. The number of distinct DRB1 alleles identified is continually increasing. The 1989 WHO Nomenclature Committee report listed 34 DRB1 alleles; this set of alleles is herein referred to as the 1989 allele set. The 1990 WHO Nomenclature Committee 5 report listed 45 alleles; this set of alleles is herein referred to as the 1990 allele set. In addition, the present invention provides the sequences of several newly discovered alleles.

The diversity of DRB loci, the most highly polymorphic of the HLA Class II loci, and the large number of alleles at these loci in the population make difficult the 10 process of identifying the particular DRB loci and alleles from which a nucleic acid in a sample originates. The present invention allows one to make this determination with great specificity and so can be used to identify the particular individual from whom a sample was taken. This discrimination power in turn leads to the applications of the invention in the field of forensic science.

15 Because PCR (or other amplification processes) can be used to amplify very small amounts of DNA (or degraded DNA), the present invention can be used to type HLA DRB DNA from unusual sources, such as a buccal swab, a single hair, and even DNA from preserved ancient specimens. With the latter samples, analysis of the alleles from prehistoric sources, e.g., early hominids, is possible. For purposes of the 20 present invention, "amplification" is defined by any process that increases the amount of target nucleic acid in a sample by means of nucleic acid replication or transcription.

The present method can be used for DRB DNA typing of cells that do not express the DRB genes. The method is also suitable, however, for DRB DNA typing of cDNA synthesized from DRB mRNA. The latter method facilitates the study of the 25 expression of HLA DRB in various cell lines or tissues and can be used to determine if there is an association between HLA DRB expression and susceptibility to transformation, autoimmunity, or other health conditions.

The research potential of the present invention should in no way obscure the immediate clinical applications. The genes and gene products of the MHC play a 30 central role in the immunological state of an individual, and particular MHC gene products are associated with disease resistance and susceptibility. Because the present invention allows the determination of the MHC DRB gene products in a sample, the invention also has applications in the field of medicine, particularly for medical diagnostic methods.

35 The discriminating power of this system will be valuable in typing potential transplantation donors, where very precise HLA DRB matching appears to be critical in minimizing risk of rejection or graft versus host disease. See the article entitled "Mixed lymphocyte reactions for individuals with phenotypic identity for specific HLA B, DR

determinants: The role of linkage disequilibrium and of specific DR and other Class II determinants" by Pollack et al., 1983, *J. Clin. Immunol.* 3:341. Disease susceptibility studies have shown that single nucleotide differences in the DRB alleles can be medically significant. See the articles entitled "Specific HLA-DQB and HLA-DRB1 alleles confer susceptibility to *Pemphigus vulgaris*" by Scharf et al., 1989, *Proc. Natl. Acad. Sci. USA* 86:6215; and "Identification of HLA-Dw14 genes in DR4+ Rheumatoid Arthritis" by Nepom et al., 1986, *Lancet* page 1002.

In addition to the above benefits, the present invention also provides methods for identifying previously unknown DR alleles, and related primers, probes, and methods for the identification of any DRB allele. Unusual patterns of SSO probe hybridization using this typing system identify new alleles, as exemplified by the DNA typing of the "Beguit" cell line (see Example 7). This cell line showed an unusual pattern of probe hybridization at the DRB1 locus, revealing a previously unreported sequence, now designated DRB1*1103 (SEQ ID NO: 27).

In similar fashion, analysis by the present method of the DRB1 alleles in a patient with Lyme disease revealed a new pattern of probe hybridization. DRB1 allele sequences were cloned from genomic DNA of the patient and sequenced. The sequence of one DRB1 allele of the patient is different from any previously reported DRB1 allele and was originally designated as DR'LY10' (also DRB1*LY10). The other allele in the patient was DRB1*0402. The DR'LY10' allele has regions of sequences similar to DRB1*0801, *0802, *0803, and *1201 at the 5'-end and to *1401 at the 3'-end. The present invention provides probes for distinguishing the DR'LY10' allele from other DRB alleles. The DR'LY10' allele is now designated as DRB1*1404 (SEQ ID NO: 40).

Other newly discovered alleles of the DRB1 locus are DRB1*1305 (SEQ ID NO: 36), originally designated as DR'PEV', DRB1*1303 (SEQ ID NO: 34), and DRB1*1105 (SEQ ID NO: 29), originally designated as DRB1*BUGS. Each was discovered when analysis by the methods of the present invention revealed a new pattern of probe hybridization. Subsequent sequence analysis of each allele revealed the sequence variation causing the novel hybridization pattern. The present invention provides primers and probes for distinguishing the newly discovered alleles from other DRB alleles.

The present invention also provides kits for making practice of the present DRB typing method more convenient. One type of kit includes both amplification and typing reagents. Another kit contains only one or more DRB probes of the invention. In either kit, the probes can be labeled or unlabeled or attached to a solid support. The primers, if present in the kit, can also be labeled to facilitate detection, i.e., to bind a signal development reagent or for immobilization. The kits can also contain reagents

that facilitate detection of probe hybridization, i.e., the chromogenic substrate TMB and streptavidin-linked horseradish peroxidase. In brief, the reagents useful in practicing the present method can be packaged in any configuration that promotes utilization of the invention.

- 5 The nucleotide sequence of the second exon of each allele along with the encoded amino acid sequence are provided in the Sequence Listing section. Table 1 and 2, below, presents equivalent nucleotide sequence information for the alleles in the 1989 allele set, but in a manner which facilitates comparison. Table 1 also shows the nucleic acid sequence and the encoded amino acid sequence in both three and one letter
- 10 codes for allele DRB*0101. Similarly, Table 3 presents equivalent amino acid sequence information, but using one letter amino acid codes. The sequence identification number of each allele is shown below. All alleles with the exception of DRB1*1503, DRB1*0303, and DRB1*1105 are listed in the 1990 WHO Nomenclature Committee report, *supra* (1990 allele set). For the sequence of
- 15 DRB1*1503, see also Demopulos et al., 1991, *Human Immunology* 30:41-44.

The nucleotide sequences of alleles which do not appear in Tables 1, 2, and 3 are provided in Table 9 below in addition to the Sequence Listing section.

| | <u>Allele</u> | <u>SEQ ID NO:</u> | <u>Allele</u> | <u>SEQ ID NO:</u> |
|----|---------------|-------------------|---------------|-------------------|
| 20 | DRB1*0101: | SEQ ID NO: 1 | DRB1*1201: | SEQ ID NO: 30 |
| | DRB1*0102: | SEQ ID NO: 2 | DRB1*1202: | SEQ ID NO: 31 |
| | DRB1*0103: | SEQ ID NO: 3 | DRB1*1301: | SEQ ID NO: 32 |
| | DRB1*0301: | SEQ ID NO: 4 | DRB1*1302: | SEQ ID NO: 33 |
| | DRB1*0302: | SEQ ID NO: 5 | DRB1*1303: | SEQ ID NO: 34 |
| 25 | DRB1*0303: | SEQ ID NO: 6 | DRB1*1304: | SEQ ID NO: 35 |
| | DRB1*0401: | SEQ ID NO: 7 | DRB1*1305: | SEQ ID NO: 36 |
| | DRB1*0402: | SEQ ID NO: 8 | DRB1*1401: | SEQ ID NO: 37 |
| | DRB1*0403: | SEQ ID NO: 9 | DRB1*1402: | SEQ ID NO: 38 |
| | DRB1*0404: | SEQ ID NO: 10 | DRB1*1403: | SEQ ID NO: 39 |
| 30 | DRB1*0405: | SEQ ID NO: 11 | DRB1*1404: | SEQ ID NO: 40 |
| | DRB1*0406: | SEQ ID NO: 12 | DRB1*1405: | SEQ ID NO: 41 |
| | DRB1*0407: | SEQ ID NO: 13 | DRB1*1501: | SEQ ID NO: 42 |
| | DRB1*0408: | SEQ ID NO: 14 | DRB1*1502: | SEQ ID NO: 43 |
| | DRB1*0409: | SEQ ID NO: 15 | DRB1*1503: | SEQ ID NO: 44 |
| 35 | DRB1*0410: | SEQ ID NO: 16 | DRB1*1601: | SEQ ID NO: 45 |
| | DRB1*0411: | SEQ ID NO: 17 | DRB1*1602: | SEQ ID NO: 46 |
| | DRB1*0701: | SEQ ID NO: 18 | DRB2*0101: | SEQ ID NO: 47 |
| | DRB1*0801: | SEQ ID NO: 19 | DRB3*0101: | SEQ ID NO: 48 |

| | | | | |
|----|------------|---------------|------------|----------------|
| | DRB1*0802: | SEQ ID NO: 20 | DRB3*0201: | SEQ ID NO: 49 |
| | DRB1*0803: | SEQ ID NO: 21 | DRB3*0202: | SEQ ID NO: 316 |
| | DRB1*0804: | SEQ ID NO: 22 | DRB3*0301: | SEQ ID NO: 50 |
| | DRB1*0901: | SEQ ID NO: 23 | DRB4*0101: | SEQ ID NO: 51 |
| 5 | DRB1*1001: | SEQ ID NO: 24 | DRB5*0101: | SEQ ID NO: 52 |
| | DRB1*1101: | SEQ ID NO: 25 | DRB5*0102: | SEQ ID NO: 53 |
| | DRB1*1102: | SEQ ID NO: 26 | DRB5*0201: | SEQ ID NO: 54 |
| | DRB1*1103: | SEQ ID NO: 27 | DRB5*0202: | SEQ ID NO: 55 |
| | DRB1*1104: | SEQ ID NO: 28 | | |
| 10 | DRB1*1105: | SEQ ID NO: 29 | | |

In Tables 1, 2, and 3, below, all sequences except the recently identified "PEV" and "LY10" alleles are listed in the 1989 WHO HLA nomenclature report, *supra*, and the DRB1*0101 nucleotide sequence serves as the consensus sequence. The inferred 15 amino acid sequence for the consensus sequence is written in one and three letter code above the nucleotide sequence. Sequence homology is indicated by dashed lines, and letters indicate polymorphic bases. The designated SSO probes and primers written at the right end of the alignments in the tables are the same sense as, or complementary to, the regions of sequences boxed. Where two names appear at the end of an alignment, 20 the left-most name refers to the left-most box, the right-most name refers to the right-most box. The probe CRX12 hybridizes to the region shown in all DRB alleles.

Table 1, in three parts designated A, B, and C, shows the nucleotide sequence of 35 DRB1 alleles corresponding to the DR specificities DR1 to DRw18.

Table 2 shows the nucleotide sequence alignments for the alleles of the DRB2, 25 DRB3, DRB4, and DRB5 loci. The major regions of sequence polymorphism for the DRB1 alleles are localized to amino acid positions 9-16, 25-34, 67-74, and 86; the remainder of the second exon sequence is relatively invariant.

Table 3 shows the inferred amino acid sequence alignment encoded by the DRB alleles. Analysis of the amino acid sequences reveals a complex but restricted pattern of 30 polymorphism with particular polymorphic sequences found in several different alleles. However, some polymorphic sequences are unique for each allele. The DR1, DR2, DR4, DR7, DRw9, and DRw10 alleles each have unique polymorphic sequences at the first hypervariable region (positions 9 to 16) of the DRB1 locus that can be used to determine the serological DR specificities by SSO typing. By contrast, the DR3, 35 DRw11, and DRw6 alleles share a polymorphic epitope, "YSTS," and cannot be distinguished by a probe for this region alone but can be distinguished by the polymorphisms at other positions in each allele. Similarly, DRw8 and DRw12 also cannot be distinguished in this region.

Table 1A

Table 1B

Table 1C

Table 2

Table 3

ALIGNMENT OF HLA-DR1 PROTEIN SEQUENCES

| | 10 | 20 | 30 | 40 | 50 | 60 | 70 | 80 | 90 |
|------------------|-----|--------|-----|----------|-----|----------|-----|---------|-----|
| CONSENSUS | | | | | | | | | |
| DRB1*0101: | R | F | I | E | O | K | *S | C | H |
| DRB1*0102: | --- | N | -L- | F | --- | L-E-CI-N | --- | S | --- |
| DRB1*0103: | --- | N | -L- | F | --- | L-E-CI-N | --- | S | --- |
| DRB1*0104: | --- | N | -L- | F | --- | L-E-CI-N | --- | S | --- |
| DRB1*1501: | --- | N-P-R | --- | N-S | --- | F | --- | I-A | --- |
| DRB1*1502: | --- | N-P-R | --- | N-S | --- | F | --- | I-A | --- |
| DRB1*1601: | --- | N-P-R | --- | N-S | --- | F | --- | F-DR | --- |
| DRB1*1602: | --- | N-P-R | --- | N-S | --- | F | --- | DR | --- |
| DRB1*0301: | --- | YSTS | --- | Y | --- | HN | --- | N | --- |
| DRB1*0302: | --- | YSTS | --- | E | --- | HN | --- | V | --- |
| DRB1*0401: | --- | V-H | --- | H | --- | K | --- | K-GR | --- |
| DRB1*0402: | --- | V-H | --- | H | --- | K | --- | DR4-DR4 | --- |
| DRB1*0403: | --- | V-H | --- | H | --- | I-DE | --- | V | --- |
| DRB1*0404: | --- | V-H | --- | H | --- | R-E | --- | V | --- |
| DRB1*0405: | --- | V-H | --- | H | --- | R | --- | V | --- |
| DRB1*0406: | --- | V-H | --- | H | --- | S | --- | V | --- |
| DRB1*0407: | --- | V-H | --- | H | --- | R-E | --- | V | --- |
| DRB1*0408: | --- | V-H | --- | H | --- | R | --- | V | --- |
| DRB1*1101: | --- | YSTS | --- | H | --- | F | --- | F-DR | --- |
| DRB1*1102: | --- | YSTS | --- | N | --- | E | --- | I-DE | --- |
| DRB1*1103: | --- | YSTS | --- | N | --- | E | --- | V | --- |
| DRB1*1104: | --- | YSTS | --- | N | --- | F | --- | V | --- |
| DRB1*1105: | --- | YSTS | --- | N | --- | F | --- | V | --- |
| DRB1*1302: | --- | YSTS | --- | HN | --- | F | --- | I-DE | --- |
| DRB1*1303: | --- | YSTS | --- | HN | --- | F | --- | I-DK | --- |
| DRB1*1401: | --- | YSTS | --- | HN | --- | A-H | --- | RR | --- |
| DRB1*1402: | --- | YSTS | --- | HN | --- | E-H | --- | E | --- |
| "DR PEV" | --- | YSTS | --- | HN | --- | F | --- | F-DR | --- |
| "LX10" | --- | YSTS | --- | HN | --- | A-H | --- | RR-E | --- |
| DRB1*0701: | --- | H-G-YK | --- | Q-E-L-N | --- | V-S | --- | I-DR-GO | --- |
| DRB1*0801: | --- | YSTS | --- | N | --- | S | --- | I-DR | --- |
| DRB1*0802: | --- | YSTS | --- | N | --- | F-DR | --- | L | --- |
| DRB1*0803: | --- | YSTS | --- | N | --- | F-DR | --- | L | --- |
| DRB1*0901: | --- | K-D-F | --- | Y-H-GI-N | --- | N | --- | S | --- |
| DRB1*1001: | --- | EV-T | --- | L-E-RVHN | --- | A-Y | --- | RR | --- |
| DRB1*1201: | --- | YSTG-Y | --- | L-E-H-HN | --- | LL | --- | V-S | --- |
| | | | | | | 1 | -DR | -AV | --- |

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Table 3 - Continued

| | |
|-------------|--|
| CONSENSUS : | RFLEQ*K*ECHFFFNGTERVRFDRYFY*QEEYVRFDSDVGEYRAVATELGRPDAEYWNSQKDILEQ*RAAVDTICRHNYGVGESFTVQRR |
| DRB2*0101 : | ---A-C---I---MK---QY-N--IHKR--NLP-----E-FQ-----V--N-----GI---EN-DK--- |
| DRB3*0101 : | ---LR-S-----Y-----HN-----FL-----V-----S-----K-GR-N-----DRw52a |
| DRB3*0201 : | ---LL-S-----E-H-HN-----A-----R-----K-GQ-N-----DRw52b |
| DRB3*0301 : | ---LL-S-----E-HN-----F-----V-----S-----K-GQ-N-----DRw52c |
| DRB4*0101 : | ---A-C----I-----W-N-I--I-N-----A-YN-----L-----Q-----RR-----E-----Y-----V----- |
| DRB5*0101 : | ---Q-D-Y-----H-DI-N-----DL-----F-----DR-----DR2-Dv2 |
| DRB5*0102 : | ---Q-D-Y-----H-GI-N-----N-----F-----DR-----DR2-Dv12 |
| DRB5*0201 : | C---Q-D-Y-----H-GI-N-----N-----I-----A-----AV-----DR2-Dv21 |

The classical serologically defined DR types (except DR3 and DRw6) can be distinguished by amplifying with the generic DRB primers GH46 (SEQ ID NO: 67) and GH50 (SEQ ID NO: 68) and analyzing the amplification products with the first panel of probes, shown in Table 4. The allele specificities shown in Table 4 are with respect to the 1989 allele set. For purposes of the present invention, "generic primers" are PCR primers that hybridize to DRB gene second exon sequences and can be used to amplify any allele of any DRB locus. Because of the hybridization site of GH50, one cannot probe for polymorphisms at position 86 (referring to the amino acid sequence) of the second exon when the product of amplification was generated with primer GH50. An alternate primer that can be used to generate products than can be analyzed for position 86 polymorphisms is DRB151 (SEQ ID NO: 227),
 10 5'-CCGAATTCGCCGCTGCACTGTGAAGCT-3'.

Table 4
First Panel of HLA DRB Typing SSO Probes

| | | | <u>HLA DRB</u> | |
|----|-------|---------------|------------------------|--|
| | | | <u>Alleles (DRB1*)</u> | <u>Wash (SSPE, °C.)</u> |
| 15 | Probe | SEQ ID NO: | Epitope | |
| | CRX60 | SEQ ID NO: 79 | "W-L-F" | 0101, 0102, 0103 0.4X, 42 |
| | GH105 | SEQ ID NO: 91 | "Q-D-Y" | DRB5 0.1X, 42 |
| | GH104 | SEQ ID NO: 90 | "W-P-R" | 1501, 1502, 1601, 1602 0.2X, 42 |
| 20 | GH59 | SEQ ID NO: 87 | "V-H" | 0401-0408 0.2X, 42, 20' |
| | CRX06 | SEQ ID NO: 61 | "I-DE" | 0103, 0402, 1102, 1301, 1302 0.1X, 42 |
| | GH122 | SEQ ID NO: 93 | "E" | 1101, 1102, 1103, 1104 0.2X, 42 |
| | CRX23 | SEQ ID NO: 66 | "A-H" | 1401, DR "LY10" 0.1X, 42 |
| 25 | CRX35 | SEQ ID NO: 71 | "F-DR" | 1601, 1101, 1104, DR "PEV", 0801, 0802 0.2X, 42 |
| | CRX49 | SEQ ID NO: 74 | "G-YK" | 0701, 0702 1.0X, 42 |
| | GH102 | SEQ ID NO: 89 | "YSTG" | 0801, 0802, 0803, 1201, DR "LY10", 1404 0.1X, 42 |
| 30 | GH111 | SEQ ID NO: 92 | "K-D-F" | 0901 0.4X, 42 |
| | CRX34 | SEQ ID NO: 70 | "EV" | 1001 0.4X, 42 |
| | CRX04 | SEQ ID NO: 60 | "R" | 0101, 0102, 0403, 0404, 0405, 0406, 0407, 0408, 1402 0.1X, 42 |
| 35 | GH56 | SEQ ID NO: 86 | "YSTS" | 0301, 0302, 1101-1104, 1301-1303, 1401, 1402, DR "PEV" 0.2X, 42 |
| | CRX68 | SEQ ID NO: 84 | "F-DE" | 1103 0.2X, 42 |
| | CRX12 | SEQ ID NO: 63 | DRB "ALL" | All HLA-DRB alleles 0.2X, 42 |

The probes shown in Table 4 are hybridized and then washed for 15 minutes at 42°C, except for GH59, which is washed for 20 minutes at 42°C. All SSPE wash solutions contain 0.1% SDS. Each probe is conjugated to HRP at the 5'-end.

- This PCR/SSO DRbeta typing system is useful for "subtyping" the serologically defined DR haplotypes. For example, the cell line "KOSE" is homozygous for DRw6 when typed serologically; however, PCR/SSO DRbeta typing reveals that "KOSE" has two different DRw6 alleles, DRB1*1302 and DRB1*1401.

- Samples that may be DR3 or DRw6 can be distinguished by amplifying with the DRB1 specific primers GH46 and CRX37 and analyzing the amplification products with the probes GH125 and CRX50 from the second panel of probes, shown in Table 5. The allele specificities shown in Table 5 are with respect to the 1989 allele set.

Table 5
Second Panel of HLA DRB Typing SSO Probes

| HLA DRB | | | | | |
|---------|--------------------|---------------|-------------------------------|---|------------------|
| 15 | Probe | SEQ ID NO: | Epitope | Alleles (DRB1*) | Wash (SSPE, °C.) |
| | GH125 ^a | SEQ ID NO: 94 | "Y" | 0301 | 0.2X, 50 |
| | CRX50 ^a | SEQ ID NO: 75 | "K-GR" | 0301, 0302 | 0.2X, 50 |
| | CRX53 ^a | SEQ ID NO: 76 | "K" | 0401 | 0.5X, 55 |
| | CRX15 ^a | SEQ ID NO: 64 | "R-E" | 0403, 0406, 0407 | 0.4X, 55 |
| 20 | CRX62 | SEQ ID NO: 81 | "I-DK" | 1303 | 0.2X, 42 |
| | CRX63 | SEQ ID NO: 82 | "I-DR" | 0803, 1201 | 0.2X, 42 |
| | CRX61 | SEQ ID NO: 80 | "S" | 0405, 1303, 0801, 0803 | 0.1X, 42 |
| | GH54 ^a | SEQ ID NO: 85 | "V-S" | 0701, 0901, 1201 | 0.2X, 42 |
| 25 | CRX56 ^a | SEQ ID NO: 86 | "G" | 0101, 0103, 0302, 0401, 0405, 0407, 0408, 1101, 1302, 1303, 0701, 0901, 1001, 1402, DR "PEV", 0801, 0802, 0803, 1502, 1601, 1602 | 0.2X, 42 |
| 30 | CRX57 ^a | SEQ ID NO: 78 | "V" | 0301, 0402, 0403, 0404, 0406, 1102, 1103, 1104, 1301, DR "LY10," 1401, 1501 | 0.1X, 42 |
| | CRX12 | SEQ ID NO: 63 | DRB "ALL" All HLA DRB alleles | | 0.2X, 42 |

- 35 For the probes shown in Table 5, all SSPE wash solutions contain 0.1% SDS. In addition, probes marked "a" require DRB1 specific amplification with primers GH46/CRX37. CRX15 is hybridized at 50°C instead of 42°C. Each probe is conjugated to HRP at the 5'-end. The probes CRX56 and CRX57 do not completely base-pair with the epitope "G" and epitope "V" alleles, respectively, because the probes

contain one fewer G residue than the alleles. Probes modified to include the G residue missing in CRX56 and CRX57 are within the scope of the present invention.

For purposes of the present invention, "DRB1 specific" primers are primer pairs that comprise at least one primer that hybridizes to an intron sequence flanking the second exon of the DRB1 gene, which primer will not hybridize to the other DRB genes. Table 6 shows the nucleotide sequence of the DRB1 and DRB3 introns of the DR3 haplotype, from the last 10 codons prior to the start of the 3' downstream intron. The sequence for CRX37 (SEQ ID NO: 73) is underlined; the solid arrow indicates the direction of extension of the primer. The asterisks indicate sequence differences between the DRB1 and DRB3 intron sequences. The segments of the 3' downstream introns are listed in the Sequence Listing as SEQ ID NO: 314 (DRB1) and SEQ ID NO: 315 (DRB3); sequences upstream of the intron are in the allele sequences.

Table 6

| | | 85 | 90 | |
|----|----------|---|-------------------------------|-----------|
| 15 | AA Seq: | Val Val Glu Ser Phe Thr Val Gln Arg Arg ← | <u>TGGCGCCGCCCTTAAG</u> | CRX37 |
| | DRB1 NT: | GTT GTG GAG ACC TTC ACA GTG CAG CGG CGA | GGTGAGCGCGCGCGCGGGGGGGGG..... | *** * * * |
| | DRB3 NT: | GTT GTG GAG ACC TTG ACA GTG CAG CGG CGA | GGTGAGCATGTCGGGGGGCGG..... | |
| 20 | Intron | <u>Seq. ID No.</u> | <u>Sequence</u> | |
| 20 | DRB1 | SEQ ID NO: 314 | 5'-GGTGAGCGCGCGCGCGGGGG | |
| | DRB3 | SEQ ID NO: 315 | 5'-GGTGAGCATGTCGGGGGGCGG | |

The DR3, DR4, and DRw6 haplotypes appear to be evolutionarily distinct from DR2, DR7, and DR9 haplotypes. There may be differences in the intron sequences of these alleles such that the primer is too mismatched to prime extension. Because the DRB1 alleles may have diverged in evolution prior to gene duplication, it may prove difficult to find DRB1 specific primer sequences for amplifying the DRB1 gene second exon from all DR haplotypes.

The HLA DRB1 alleles can be distinguished with greater specificity by amplifying with the generic and DRB1 specific primers and analyzing with both panels (Tables 4 and 5) of probes as required. The alleles of the 1989 allele set that cannot be distinguished with the specific probes in Tables 4 and 5 are the four DR2 subtypes DRB1*1501, *1502, *1601, and *1602 and the DR4 subtypes DRB1*0403 and DRB1*0406.

The DRB1 specific PCR primers are capable of reproducibly amplifying the DRB1 sequences from all but the DR2, DR7, and DR9 haplotypes. The primer CRX37 was designed from intron nucleotide sequences from DR3, DR4, and DRw6 haplotypes. See the article entitled "Sequence Analysis of HLA Class II Genes from Insulin-Dependent Diabetic Individuals" by Horn *et al.*, 1988, Hum. Immunol. 1:249.

The present invention provides a number of allele specific and group specific primers in addition to the DRB1 specific primer pair GH46/CRX37. These primers are shown below. For the sequence of AB60, see Todd et al., 1987, Nature 329:599.

| | | | |
|------|---------------|--------------------------------|------------------------------------|
| AB54 | SEQ ID NO: 56 | 5'-GGGGATCCTGGAGCAGGTTAAACA-3' | |
| 5 | AB60 | SEQ ID NO: 57 | 5'-CCGAATTCCGCTGCACTGTGAAGCTCTC-3' |
| | AB82 | SEQ ID NO: 58 | 5'-GGGGATCCTGGAGTACTCTACGTC-3' |
| | AB83 | SEQ ID NO: 59 | 5'-GGGGATCCTGTGGCAGCCTAAGAGG-3' |

For example, DR4 specific amplification can be achieved with PCR primer pair AB54/AB60 (PCR profile: 35 cycles of: ramp to 94°C; 30 seconds denaturing at 94°C; 10 30 seconds annealing and extending at 65°C). DR3, DR5, and DR6 group specific amplification can be achieved with PCR primer pair AB82/AB60 (35 cycles of: ramp to 96°C; 30 seconds denaturing at 96°C; 30 seconds annealing and extending at 65°C). DR2 specific amplification can be achieved with PCR primer pair AB83/AB60 (35 cycles of: ramp to 96°C; 30 seconds denaturing at 96°C; 30 seconds annealing and 15 extending at 70°C).

Specific alleles can be detected after group specific amplification with the epitope specific probes described herein; for instance, DRB1*1601 can be detected after DR2 specific amplification with the AB83/AB60 primer pair with an "F-DR" epitope specific probe.

20 Detection of alleles of the DR2 haplotype can be difficult, but such difficulty can be overcome by the group specific primers and the typing methods of the present invention. In the generic primer amplification of DRB alleles from DR2 haplotypes, both the DRB1 and DRB5 loci are amplified. Although one could use an "F" (at position 47) epitope specific DNA probe to determine the serologic DRw15 and 25 DRw16 subtypes, "F" occurs also in O301, some DRw13 alleles, and in all DRw11 and DRw12 alleles; other methods can provide more complete discrimination. Thus, a group specific primer designed to hybridize to the sequence encoding the epitope "I-A" will amplify the DRB1*1501 and DRB1*1502 alleles, and the amplified product will hybridize with a "WPR" epitope specific DNA probe. The primer DRB150 (SEQ ID 30 NO: 226) (5'-TGTCCACCGCGGCCGCGCCT-3') is a primer designed to perform such an allele specific amplification (with GH46). The DRB5*0201 and DRB5*0202 alleles will also amplify with a group (or epitope) specific "I-A" primer, but as these alleles occur only with the DRB1*1601 and DRB1*1602 alleles, the result of probe hybridization will be epitope "QDY" positive and "WPR" negative.

35 In addition, one should note that because the DRB1 specific primers exemplified herein do not amplify DR2 alleles, the DR2 specific amplification described above can be carried out in the same reaction tube and at the same time as the DRB1

specific amplification. Other allele and group specific primers and amplification methods are described in the Examples.

The extensive allelic diversity at the HLA DRB loci, like that of the other Class II beta genes, is localized primarily to the second exon. In general, the pattern of 5 second exon sequence polymorphism, when present in the population at a particular DRB locus, is a patchwork, with specific polymorphic segments found in a variety of different alleles. In principle, such shared epitopes among different alleles could reflect either common ancestry, gene conversion, or convergent evolution. For purposes of oligonucleotide typing, however, this patchwork pattern of polymorphism means that 10 many alleles cannot be identified by hybridization to a single oligonucleotide but can be identified by a unique pattern of hybridization with a panel of probes.

The sequence-specific oligonucleotide probes of the invention, called "SSOs," when employed in the present methods under the appropriate hybridization and wash conditions, are extremely specific, capable of distinguishing single nucleotide 15 polymorphisms. The SSOs of the invention, unlike the probes used in RFLP methods, can be used to determine not only if alleles are different but also where and how the alleles differ.

In a preferred embodiment, these probes comprise the same sequence as, or a sequence complementary to, the regions of DNA of each allele boxed in Tables 1 and 2. 20 For example, the probe CRX60 is specific for the DNA encoding the amino acids "W-L-F" and can hybridize uniquely to the DR1 allele. The probe CRX49 is specific for the amino acids encoding "G-YK" and hybridizes to the DR7 allele; other examples will be apparent upon consideration of the Tables.

While a single probe (i.e., GH56) can be used to distinguish DR3, DRw11, 25 and DRw6 from the other DR serotypes, additional probes are required to distinguish between DR3, DRw11, and DRw6. The GH56 + GH122 probe combination, which hybridizes to DNA encoding the "YSTS" and "E" epitopes (codons 10 to 13 and 58) on DRw11, can be used to distinguish DRw11 from DR3 and DRw6. Likewise, the probe combinations GH56 + CRX06 corresponding to the "YSTS" and "I-DE" (codon 30 67 to 71) epitopes can be used to distinguish DRw6 (DRB1*1301 and DRB1*1302 but not DRB1*1303, which has the epitope "I-DK" but is still DRw6) from DRw11 and DR3.

The combinations of the SSO probes shown in Table 4 used to determine the majority of the serological DR types are shown in Table 7.

Table 7Combinations of SSO Probes to Determine Serological DR Types

| | <u>DR Type</u> | <u>Probe(s)</u> |
|----|--------------------|----------------------|
| 5 | DR1 Dw1 | CRX60 + CRX04 |
| | DR1 Dw "BON" | CRX60 + CRX06 |
| | DR2 | GH105 |
| | DR4 Dw4 | GH59 + CRX53 |
| | DR4 Dw10 | GH59 + CRX06 |
| 10 | DR4 Dw13, 14, 15 | GH59 + CRX04 |
| | DR3, w11, w6 | GH56 |
| | DRw11.1 | GH56 + GH122 + CRX35 |
| | DRw11.2 | GH56 + GH122 + CRX06 |
| | DRw12, DRw8.3 | GH102 + CRX63 |
| 15 | DRw13 (1301, 1302) | GH56 + CRX06 |
| | DRw14 Dw16 | GH56 + CRX04 |
| | DRw14 Dw9 | GH56 + CRX23 |
| | DR7 | CRX49 |
| | DRw8.1, w 8.2 | GH102 + CRX35 |
| 20 | DR9 | GH111 |
| | DRw10 | CRX34 |
| | DRw12, all DRw8 | GH102 |

Further subdivision of the DR serotypes (Table 4) requires the use of probes shown in Table 5, as shown in Table 8, below.

Table 8Combinations of SSO Probes to Distinguish 31 of 34 HLA DRB1 Alleles (1989 set)

| | <u>DRB1*</u> | <u>Probe(s)</u> | <u>DRB1*</u> | <u>Probe(s)</u> |
|----|--------------|------------------------------|--------------|--|
| 5 | 0101 | CRX60 + CRX04 + CRX56 | 1103 | GH122 + GH56 + CRX57 + CRX68 |
| | 0102 | CRX60 + CRX04 | 1104 | GH122 + GH56 + CRX35 + CRX57 |
| | 0103 | CRX60 + CRX06 + CRX56 | 1201 | GH102 + GH54 + CRX63 |
| | DR2 | GH105 | 1301 | GH56 + CRX06 + CRX57 |
| | 0301 | GH56 + GH125 + CRX50 + CRX57 | 1302 | GH56 + CRX06 + CRX56 |
| | 0302 | GH56 + CRX50 + CRX56 | 1303 | GH56 + CRX62 + CRX61 + CRX56 |
| 10 | 0401 | GH59 + CRX53 + CRX56 | 1401 | GH56 + CRX23 + CRX57 |
| | 0402 | GH59 + CRX06 + CRX57 | 1402 | GH56 + CRX04 + CRX56 |
| | 0403, 0406 | GH59 + CRX15 + CRX57 + CRX04 | DR "PEV" | GH56 + CRX35 + CRX56 |
| | 0404 | GH59 + CRX04 + CRX57 | 0701 | CRX49 + GH54 + CRX56 |
| | 0405 | GH59 + CRX04 + CRX61 + CRX56 | 0801 | GH102 + CRX61 + CRX35 + CRX56 |
| 15 | 0407 | GH59 + CRX15 + CRX56 + CRX04 | 0802 | GH102 + CRX35 + CRX56 |
| | 0408 | GH59 + CRX04 + CRX56 | 0803 | GH102 + CRX61 + CRX63 + CRX56 |
| 20 | 1101 | GH122 + GH56 + CRX35 + CRX56 | 0901 | GH111 + GH54 + CRX56 |
| | 1102 | GH122 + GH56 + CRX06 + CRX57 | 1001 | CRX34 + CRX56 DR "LY10" GH102 + CRX23 + CRX57 |

The horseradish peroxidase-conjugated SSOs of the invention, called "HRP-SSOs," allow detection methods that employ chromogenic or chemiluminescent substrates that are easy to use and produce detectable signals rapidly (typically 1 to 10 minutes). The HRP-SSOs are stable for over two years without detectable loss of activity when stored at 4°C. See the article entitled "Nonisotopically labeled probes and primers" by Levenson and Chang, 1989, in PCR Protocols: A Guide to Methods and Applications (Innis, Gelfand, Sninsky and White ed., Academic Press, Inc. San Diego). Radiolabelled probes can be employed but are not necessary for excellent sensitivity, an important benefit provided by the present invention.

The dot-blot format for detection enables the rapid typing of a large number of samples and will be useful in determining the allele frequencies of HLA DRB. A recently developed alternative for PCR/SSO DRbeta typing is the immobilized reverse dot-blot format. See the article entitled "Genetic analysis of amplified DNA with immobilized sequence-specific oligonucleotide probes" by Saiki *et al.*, 1989, Proc. Natl. Acad. Sci. USA 86:6230, and copending Serial No. 347,495, filed May 4, 1989, incorporated herein by reference. In this procedure, the SSO probes are applied and

fixed to the filter (rather than the amplified DNA applied and fixed to the filter), hence the term "reverse dot blot."

The reverse dot blot procedure allows a single sample to be analyzed in a single hybridization with a membrane containing an array of immobilized probes. The conventional dot blot format is useful when the number of samples exceeds the number of probes used (e.g., patient versus control or population genetics studies). The reverse dot blot format is valuable for clinical, diagnostic, and forensic analyses. The reverse dot blot format is described in more detail in Example 8.

The following examples show illustrative preferred embodiments of the present invention. The examples show that the present invention provides, in a preferred embodiment, a nonisotopic PCR/SSO system for HLA DRB typing that is simple, rapid, and capable of precise DRB typing for a variety of samples from different sources.

Example 1

Amplification and Detection Methods

For typing samples with the first panel (Table 4) of probes, 0.5 µg of human genomic DNA was amplified using reaction constituents as described in the article entitled "Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase," by Saiki *et al.*, 1988, *Science* 239:487 and in Scharf *et al.*, 1988, *Hum. Immunol.* 22:61, and Scharf *et al.*, 1989, *Proc. Natl. Acad. Sci. USA* 86:6215, incorporated herein by reference.

The HLA DRB generic PCR primers are GH46 and GH50. The sequences of these primers are shown below.

GH46 SEQ ID NO: 67 5'-CCGGATCCTCGTGTCCCCACAGCACG

25 GH50 SEQ ID NO: 68 5'-CTCCCCAACCCCGTAGTTGTGTCTGCA

The primers were present in the reaction mixture at 500 nM. These primers produce a 272 base-pair (bp) fragment and contain sequences for BamHII and PstI restriction sites for cloning the PCR product. Digestion of the amplified DNA with BamHII and PstI produces a 248 bp product due to an internal PstI site.

30 The DRB1 alleles from all haplotypes (except DR2, DR7, and DR9) were specifically amplified by the PCR primers GH46 and CRX37. The sequence of the CRX37 primer is shown below.

CRX37 SEQ ID NO: 73 5'-GAATTCCCGCGCCGCGCT

Amplification with the GH46/CRX37 primer pair produces a 297 bp fragment. Primer 35 CRX37 incorporates an EcoRI restriction endonuclease recognition sequence at the 5'-end to facilitate cloning and, in contrast to the primer pair GH46/GH50, amplification

and BamHI/EcoRI digestion with this primer pair allows isolation and analysis of the full-length PCR product.

Such isolation and analysis frequently involved the determination of the nucleotide sequence of the PCR product. For sequencing the HLA DRB alleles, 1 µg 5 of purified human genomic DNA was amplified using the primer pairs GH46/GH50 and GH46/CRX37. The amplified DNA was cloned into M13mp10 by the methods described in Scharf *et al.*, 1988, Hum. Immunol. 22:61, and Scharf *et al.*, 1986, Hum. Immunol. 23:1076, incorporated herein by reference. The inserts were then sequenced by the dideoxy chain-termination procedure described in Sanger *et al.*, 1977, 10 Proc. Natl. Acad. Sci. USA 74:5463 (see also U.S. patent application Serial No. 249,367, filed September 23, 1988, and incorporated herein by reference).

The sequences shown in Tables 1 and 2 above were generated by genomic cloning (see Horn *et al.*, 1988, Hum. Immunol. 21: 249, incorporated herein by reference), PCR amplification (see Erlich *et al.*, 1989, in Immunobiology of HLA 15 (du Pont ed., Springer-Verlag, New York) and Scharf *et al.*, 1989, Proc. Natl. Acad. Sci. USA 86: 6215, incorporated herein by reference), or from the literature (see WHO Nomenclature Committee, 1990, Immunogenetics 31:131 and Gregersen *et al.*, 1989, "First domain diversity of DR and DQ subregion alleles" in Immunobiology of HLA (du Pont, ed., Springer-Verlag, New York)).

20 Samples were amplified for 32 cycles (unless otherwise noted) using the reaction conditions described above, except that 1.25 units (rather than 2.5 units) of Taq polymerase (PECI, Norwalk, CT) were added per 100 µl of reaction volume. The cDNAs for the bladder carcinoma patients were amplified for the HLA-DRB loci as described (see Kawasaki, 1989, "Amplification of RNA" in PCR Protocols: A Guide to Methods and Applications (Innis *et al.*, eds., Academic Press, San Diego)). All 25 samples were overlaid with 100 µl of high grade mineral oil (Sigma, St. Louis, MO) to prevent evaporation. After amplification, the oil overlay was extracted with 100 µl chloroform.

30 The thermal profile for each cycle comprised incubations at the following temperatures for the indicated times: 45 seconds at 94°C (for denaturation of the DNA strands), 45 seconds at 55°C (for annealing of the primers), and 45 seconds at 72°C (for extension of the primed templates). After the last cycle, the PECI Thermal Cycler (Perkin Elmer Cetus Instruments, Norwalk, CT) was programmed to incubate the samples at 72°C for 10 minutes to ensure that the final extension was complete. Care 35 should be taken to avoid cross-contamination of samples; one must particularly guard against allowing the product of one PCR to contaminate an unamplified sample. U.S. patent application Serial No. 557,517, filed July 24, 1990, and the CIP of that

application filed November 2, 1990, both incorporated herein by reference, describe preferred methods to prevent amplification of PCR product that has been "carried over" to an unamplified sample.

After amplification, a small portion of the amplified DNA was denatured and
5 applied to and crosslinked to a series of nylon filters; each filter was then hybridized to one of the labelled probes. Each SSO probe was covalently conjugated to horseradish peroxidase (HRP) and provides a means of nonisotopic detection in the presence of a chromogenic or chemiluminescent substrate. The nucleotide sequence, the encoded amino acids (or potential epitope), and the identified DR type, as well as the wash
10 conditions for each probe are listed in Tables 4 and 5.

Thus, 5 µl of each amplified DNA sample were mixed with 100 µl of a mixture composed of 0.4 M NaOH and 25 mM EDTA, and the resulting mixture applied to BioDyne B nylon filters (Pall Corp., Glen Cove, NY) using a dot-blot manifold (Bio Rad, Richmond, CA). The filters, still in the dot-blot manifold, were rinsed with a
15 mixture of 10 mM Tris-HCl and 0.1 mM EDTA, at pH 8.0, and dried on Whatman 3MM paper. The DNA was immobilized on the nylon filter by ultraviolet irradiation at a flux of 55 mJ/cm² with a Stratalinker™ (Stratagene, La Jolla, CA) UV light box.

Unless otherwise noted, all filters were hybridized in 2X SSPE (saline sodium phosphate EDTA), 5X Denhardt's solution, and 0.5% SDS with 2 pmoles of HRP-
20 SSO probe per ml of hybridization solution for 15 min. at 42°C. Horseradish peroxidase conjugated oligonucleotides were prepared as described by Levenson and Chang, 1989, in PCR Protocols: A Guide to Methods and Applications (Innis et al., eds., Academic Press, Inc. San Diego) and Saiki et al., 1988, N. Eng. J. Med.
319:537. Filters for each probe were washed in 25 ml of the SSPE solutions at the
25 temperatures listed in Tables 4 and 5 for 15 minutes (unless noted otherwise).

After washing, filters to be developed with a chromogenic dye substrate were rinsed in PBS at room temperature for 30 minutes, then placed in 100 mM sodium citrate, pH 5.0, containing 0.1 mg/ml of 3,3',5,5'-tetramethylbenzidine (TMB) per milliliter (Fluka) and 0.0015 percent hydrogen peroxide, and incubated with gentle agitation for 5 to 15 minutes at room temperature. Developed filters were rinsed in PBS and immediately photographed. Filters that were developed with the
30 chemiluminescent detection system (ECL; Amersham, Arlington Heights, IL) were rinsed in PBS for 5 minutes and placed in the ECL solution for 1 minute with gentle agitation. Filters were then exposed to X-ray film at room temperature for 1 to 5 minutes.
35

Example 2DRB1 Specific Amplification

Several polymorphic sequences of alleles of the DRB1 locus that distinguish the various DRB1 alleles are also present on alleles of the other DRB loci (see Table III).

- 5 An example is the nucleotide sequence encoding the epitope "K-GR" (codons 71 to 74) on DR3 DRB1 alleles, where a probe to this region (CRX50) can distinguish DR3 from DRw11 and DRw6 alleles. However, this epitope is also encoded by the DRB3 allele DRw52a (DRB3*0101) and is present on some DRw6 haplotypes and some DR3 haplotypes as well. Because the PCR primers GH46/GH50 amplify all of the DRB 10 loci, a DRw6 sample that was DRw52a at the DRB3 locus would be impossible to distinguish from a DR3 sample using this probe.

The present invention solves this problem by providing PCR primers that specifically amplify only the DRB1 locus. One of these primers hybridizes to a region in the intron immediately downstream from the second exon. The intron contains 15 sequences that distinguish the DRB1 and DRB3 loci. The primer (CRX37) hybridizes specifically to the DRB1 intron sequences, and combining this primer with GH46 permits DRB1 specific amplification for most haplotypes.

To determine if the DRB1 locus is the only locus amplified with these putative DRB1 specific primers, DR2, DR3, and DR4 HTC (homozygous typing cells) DNA 20 was amplified and analyzed with these primers and SSO probes for all the DRB loci. In addition to the DRB1 locus, the DR2 haplotype has a DRB2 and DRB5 locus, the DR3 haplotype has a DRB3 locus, and the DR4 haplotype has a DRB4 locus. The results are shown in Figure 1.

To generate these results, about 200 ng of HTC DNA were amplified by the 25 generic DRB primers GH46/GH50 or by the DRB1 specific primers GH46/CRX37 and applied to filters as described in Example 1. Each filter containing amplified cell line DNA (samples 1 to 4) was hybridized with the probes shown in Figure 1. The locus to which each probe hybridizes is shown in parentheses. CRX36 hybridizes to the DRB4 locus of DR4, DR7, and DR9. CRX22 hybridizes to the DRB3 allele 30 DRw52b.

The sequence of the SSOs:

CRX36 SEQ ID NO: 72 5'-CCCGCCTCCGCTCCA

CRX22 SEQ ID NO: 65 5'-GAGCTGCTTAAGTCT

is described by Scharf *et al.*, 1989, *Proc. Natl. Acad. Sci. USA* 86:6215, incorporated 35 herein by reference. Probe GH91 (SEQ ID NO: 88, 5'-CTCCCGTTATGGATGTAT) hybridizes specifically to the DRB2 locus. This probe was hybridized as described in Example 1 and washed in 1X SSPE, 0.1% SDS

for 15 minutes at 42°C. The samples amplified are: (1) No DNA (negative control); (2) DR2 HTC "SCHU"; (3) DR3 HTC "QBL"; and (4) DR4 HTC "BSM." After the hybridization and wash steps, the probes were detected by enhanced chemiluminescence.

5 Figure 1 shows that all of the DRB loci for all three HTCs are amplified with the generic DRB primers, while the DRB1 specific primers only amplify the DRB1 loci from the DR3 and DR4 HTCs. Interestingly, for the DR2 HTC, the DRB2 locus is amplified, albeit weakly, with the DRB1 specific primers. The DRB1 specific primers amplify DRB1 sequences efficiently from all DR haplotypes tested except DR2, DR7,
10 and DR9, as shown in Figures 1 and 2. To generate the results shown in Figure 2, about 0.5 µg of genomic DNA from HTCs was amplified for HLA DRB with the DRB1 specific primers GH46/CRX37. Amplification reactions and filters were prepared as described in Example 1. Each filter strip was hybridized to one of the probes shown under the appropriate conditions of hybridization and wash stringency
15 (see Tables 4, 5, and 7). After the hybridization and wash steps, filters were developed in the chromogenic substrate TMB. The samples are: (1) DR1 HTC "KAS9003"; (2) DR2 HTC "SCHU"; (3) DR3 HTC "QBL"; (4) DR4 HTC "BSM"; (5) DR5 (DRw11) HTC "SPOO1O"; (6) DRw6 HTC "OMW"; (7) DR7 HTC "MOU"; (8) DRw8 HTC "SPACH"; (9) DR9 HTC "DKB"; and (10) DRw10 HTC "SHY".

20 The results in Figure 2 indicate that the DR7 probe CRX49 ("G-YK") does hybridize to the weakly amplified DR7 HTC in this experiment. DRB1 amplification of DR7 from heterozygous samples is typically weak. These results demonstrate that the generic and DRB1 specific primers can be used for complete DRB1 typing.

Example 3

Typing for the Serological DR Types 1 Through 10

25 About 0.5 µg of genomic DNA from HTCs was amplified for HLA DRB. All samples except for nos. 3 and 6 were amplified with the generic primers GH46/GH50. Sample nos. 3 and 6 were amplified with the DRB1 specific primers GH46/CRX37. Amplification reactions and filters were prepared as described in Example 1. Each filter strip was hybridized to one of the probes shown under the appropriate conditions of hybridization and wash stringency (see Tables 4, 5, and 7). After probing and washing, filters were developed in the chromogenic substrate TMB. The samples are:
30 (1) DR1 HTC "KAS9003"; (2) DR2 HTC "SCHU"; (3) DR3 HTC "QBL"; (4) DR4 HTC "BSM"; (5) DR5 (DRw11) HTC "SPOO1O"; (6) DRw6 HTC "OMW"; (7) DR7 HTC "MOU"; (8) DRw8 HTC "SPACH"; (9) DR9 HTC "DKB"; and (10) DRw10 HTC "SHY".
35

Figure 3 demonstrates the results of DR typing for the classical, serologically defined DR types 1 through 10 on a panel of HTCs. A single probe can be used to detect each of the classical DR types, but two different PCR primer pairs were required. For all but the DR3 and DRw6 samples, the generic PCR primers GH46/GH50 can be used. The DRB1 specific primer pair GH46/CRX37 was used for amplifying the DNA of the DR3 and DRw6 samples in this array, because the probe used to detect the "Y" epitope (GH125; codon 26) on the DR3 HTC "QBL" (DRw17) is also present on the DRB3 allele (DRw52a) of the DRw6 sample. If the standard DRB primers had been used, then the "Y" probe would have hybridized to the DRw6 sample as well.

10

Example 4

General DRB1 Typing Strategy

For typing a particular sample, the generic primers should be used for the first amplification, followed by probing of the amplified DNA with the first panel of probes (Tables 4 and 7). This panel of probes will identify the majority of the serological types; although GH56 ("YSTS") will not unequivocally distinguish DR3 from DRw6.

15 For samples that are positive with GH56, or for subtyping particular DR types (e.g., the Dw types of DR4 or the subtypes of DRw8), the second panel (Table 5) of probes should be used. As discussed above, some of the probes in the second panel require amplification with the DRB1 specific pair of PCR primers. By combining the 20 results of the probings from the first and second panels, one can distinguish 31 of the 34 DRB1 alleles in the 1989 allele set. The combinations of SSO probes used to identify these alleles is shown in Table 8. The DR2 subtypes (DRB1*1501, *1502, *1601, and *1602) are not distinguished by these primers and probes, and the DRB1*0403 and DRB1*0406 alleles are not distinguished by these probes.

25 One DR2 probe of the invention comprises sequences that hybridize to the first hypervariable region ("Q-D-Y") of the DRB5 locus. Because the DRB5 locus is present on all known DR2 haplotypes and only on those haplotypes, such a probe can be used reliably to type for DR2 in an amplification with generic DRB primers. Probe GH104 ("W-P-R") hybridizes to the first hypervariable region of the DR2 DRB1 locus 30 and hybridizes specifically to DR2 DNA when amplified with the generic primers (Figure 1) but not with the DRB1 specific primers. These two probes are identical indicators of DR2 in an amplification with generic primers.

Example 5
Subtyping DR4

- The subtyping of the DR4 specificity cannot be carried out serologically. The subtypes have been revealed by two dimensional protein gel electrophoresis and cellular typing; both methods are cumbersome and time-consuming. Dw4, Dw10, Dw13, and Dw14 differ from each other at positions 70 to 74 of the third hypervariable region of the gene. Dw15 also has a serine ("S") at position 57. Dw13 also has the same arginine residue at position 71 as Dw14 and Dw15 but is distinguished by a glutamic acid ("E") at position 74.
- As a result, the SSO probe CRX04 ("R") probe hybridizes to the Dw13, Dw14, and Dw15 alleles; these alleles have to be distinguished from each other by additional probes. The SSO probe CRX15 ("R-E") specifically distinguishes Dw13 from Dw14 and Dw15, and CRX61 ("S") specifically distinguishes Dw15 from Dw14. The "G" versus "V" polymorphism at position 86 that distinguishes various DRB1 alleles (e.g., Dw14.1 or DRB1*0404 from Dw14.2 or DRB1*0408) is detected using the CRX56 probe ("G") and the CRX57 probe ("V") (see Table 5).

HTCs with DR types 1 through 10 and the five DR4 Dw subtypes were amplified with the DRB1 specific primers GH46/CRX37, applied to six identical nylon filters, and hybridized with HRP-SSO's specific for the Dw type. The results are shown in Figure 4.

To generate the results shown in Figure 4, about 500 ng of HTC genomic DNA were amplified with the DRB1 specific primers GH46/CRX37. Each pair of filters, A and B, were hybridized with the probes shown in Figure 4. The samples are: Row A: (1) No DNA control; (2) DR1 HTC "KAS9003"; (3) DR2 HTC "SCHU"; (4) DR3 HTC "QBL"; (5) DR4 Dw4 HTC "BSM"; (6) DR4 Dw10 HTC "YAR"; (7) DR4 Dw13 HTC "JHA"; (8) DR4 Dw14 HTC "BM92"; (9) DR4 Dw15 HTC "LKT3"; (10) DR5 HTC "SPOO1O"; and (11) DRw6 HTC "OMW"; Row B: (1) No DNA control; (2) DR7 HTC "MOU"; (3) DRw8 HTC "SPACH"; (4) DR9 HTC "DKB"; and (5) DRw10 HTC "SHY".

After the hybridization and wash steps, the probes were detected by enhanced chemiluminescence. Panel A shows that all but the DR2, DR7, and DRw9 HTCs hybridized to CRX12.

Panel B shows that CRX53 hybridizes specifically to the Dw4 HTC BSM. An alternative probe for typing DR4 Dw4 is DRB163 (SEQ ID NO: 239), using the hybridization and wash conditions given in Example 9. Another alternative probe for typing DR4 Dw4 that gives a much stronger signal at lower stringencies (2X SSPE, 5X

Denhardt's, 0.5% SDS at 42°C for 15 minutes hybridization and 2X SSPE, 0.1% SDS at 42°C for 15 minutes wash) is probe CRX64, shown below.

CRX64 SEQ ID NO: 83 5'-HRP-GAGGAIAAGCIGGCC-3'

HRP is horseradish peroxidase. I is inosine, which slightly destabilizes the probe.

5 Panel C shows detection of the Dw10 HTC "YAR" with the SSO CRX06 ("I-DE"). This probe also hybridizes to the HTC "OMW", which is DRw13, and shares this polymorphism.

Panel D shows specific hybridization of CRX15, which distinguishes the Dw13 sample, JHA, from the Dw14 sample BM92 and the Dw15 sample LKT3.

10 Panel E shows hybridization of CRX04 to JHA, BM92, and LKT3, which are Dw13, Dw14, and Dw15, respectively. CRX04 also hybridizes to the DR1 HTC KAS9003, which is DRB1*0101, and shares this polymorphism ("R") with these three Dw-types. The DR4 Dw14 type is inferred from the probe hybridization pattern in which samples do not hybridize to either CRX15 ("R-E") or CRX61 ("S"; panel F),
15 but do hybridize to CRX04 ("R"). Panel F shows hybridization of CRX61 to the Dw15 HTC "LKT3".

Figure 4 shows that, in general, the present invention enables one to distinguish samples that share polymorphic regions recognized by these probes by the pattern of hybridization to the first panel of probes. For example, DR4 Dw types which are
20 positive for the "R" epitope can be distinguished from DR1 by being positive for GH59 ("V-H") and negative for CRX60 ("W-L-F"); DR4 Dw10 can be distinguished from DRw13 by being positive for GH59 and negative for GH56 ("YSTS").

Table 8 shows that the two DR4 subtypes that this system is not capable of distinguishing are the DRB1*0403 and DRB1*0406 alleles. These two alleles will give
25 the same pattern of hybridization with the two panels of probes. DRB1*0406 is identical to DRB1*0403, except for a serine residue at position 37, where DRB1*0403 has a tyrosine residue. The illustrative panels of probes shown above are not capable of detecting this polymorphism, but the use of additional probes provides complete discrimination.

30

Example 6

Subtyping DR3, DR5, DRw6, and DRw8

The subtypes for the "splits" of DR3, DR5, DRw6, and DRw8 can be identified by using probes that distinguish between the various alleles of each haplotype. For example, both DRw17 and DRw18 hybridize to the "K-GR" probe CRX50, but only
35 DRw17 hybridizes to the "Y" probe GH125, which distinguishes DRw17 from DRw18. Likewise, the DRw11 and the DRw12 alleles (which used to be grouped as

DR5) can be distinguished from each other and from the three DRw8 alleles by using a different combination of probes.

DNA amplified with the DRB1 specific PCR primers CRX37/GH46 from HTCs of the "general" serological DR types 1 through 10 along with HTCs of the 5 DR3, DR5, DRw6, DRw8 subtypes, was applied to 12 filters. Because the serological DR types of these samples were already known, only the probes necessary to determine the subtypes of these alleles were used to illustrate this aspect of the invention. The results are shown in Figure 5.

To generate the results shown in Figure 5, about 500 ng of HTC genomic DNA 10 were amplified with the DRB1 specific primers GH46/CRX37. Each pair of filters, A and B, was hybridized with the probes shown. The samples are: Row A: (1) No DNA control; (2) DR1 HTC "KAS9003"; (3) DR2 HTC "SCHU"; (4) DRw17 HTC "QBL"; (5) DRw18 HTC "RSH"; (6) DR4 HTC "BSM"; (7) DRw11 HTC "SPOO1O"; (8) DRw12 HTC "HERLUF"; (9) DRw13/DRw14 "KOSE"; (10) DRw14 HTC 15 "AMALA"; (11) DRw13 HTC "SLE"; Row B: (1) No DNA control; (2) DRw13 HTC "HAG"; (3) DR PEV "BAR P"; (4) DR7 HTC "MOU"; (5) DRw8.3 HTC "TAB"; (6) DRw8.1 HTC "ARC"; (7) DRw8.2 HTC "SPL"; (8) DR9 HTC "DKB"; and (9) DRw10 HTC "SHY". The data for the first panel of probes, which shows that GH56 ("YSTS") hybridizes to DR3, DR5, and DRw6, and GH102 ("YSTG") hybridizes to 20 DRw8 is shown in Figure 7 (see Example 7).

After the hybridization and wash steps, the bound probes were detected by enhanced chemiluminescence. By determining the pattern of probes that hybridize to each sample (see Table 8), the samples can be subtyped for specific alleles, as shown in Figure 6. In Figure 6, samples which hybridized to probes are shown with a "+" symbol. Blank cells indicate samples which did not hybridize to a particular probe. 25 Except for "KOSE and BAR P", all the samples were homozygous typing cells.

The hybridization data and DR types for the DR3, DR5, DRw6 and DRw8 samples are shown in Figure 6. The sample "KOSE" was typed serologically as homozygous for the DRw6 haplotype (though this sample has been shown to be Dw9 30 and Dw19); however, it types as DRB1*1302 (CRX06 + CRX56) and DRB1*1401(CRX23 + CRX57).

The sample "BAR P" is typed by serology and MLC as DR4 Dw10 and DRw6. Consequently, "BAR P" hybridizes to the probes CRX35 and CRX56 ("F-DR" and "G"), which types it as the newly discovered "DR PEV" DRw6 allele, and to the 35 probes CRX06 and CRX57 ("I-DE" and "V"), reflecting the presence of the DR4 Dw10 allele (DRB1*0402).

Example 7
DR Typing of Cell Lines

Many established cell lines do not express the Class II molecules, making it impossible to DR type them serologically. DNA samples from five Class II negative 5 cell lines coded as a blind panel were amplified with the generic primer pair GH46/GH50 and probed with the first panel of probes (Table 4) to establish the general serological DR types for the samples (Figure 7).

For DR typing these and other cell lines, approximately 100 ng of cell line DNA were amplified for 30 cycles with the generic HLA DRB primers GH46/GH50. Each 10 filter containing amplified cell line DNA (samples A through F) was hybridized simultaneously with a control filter (samples 1 through 11) containing HTC DNAs amplified with the generic HLA DRB primers. All filters were prepared as described in Example 1. The results are shown in Figure 7. Each pair of filters was hybridized to the SSO probes shown. The samples are: (1) No DNA control; (2) DR1 HTC 15 "KAS9003"; (3) DR2 HTC "SCHU"; (4) DR3 HTC "QBL"; (5) DR4 HTC "BSM"; (6) DR5 (DRw11) HTC "SPOO10"; (7) DRw6 HTC "OMW"; (8) DR7 HTC "MOU"; (9) DRw8 HTC "SPACH"; (10) DR9 HTC "DKB"; (11) DRw10 HTC "SHY"; (A) No DNA control; (B) Raji; (C) 616; (D) Beguit; (E) RM3; and (F) RS225.

The signal intensity for the CRX12 probe demonstrates that all of the samples 20 were equally well amplified. Raji, RM3, and RS225 hybridize to the probe CRX34, which is specific for DRw10. Sample 616 hybridizes to the probes CRX60 ("WLF") and CRX04 ("R"), which is consistent with this sample having the DR1 allele.

The cell line Beguit hybridizes to the DR7 probe CRX49 ("G-YK") and to two other probes, GH56 ("YSTS") and GH122 ("E"), suggesting that it is DR7 and 25 DRw11. However, the putative DRw11 allele from this sample would be expected to hybridize either to CRX35 ("F-DR"; DRB1*1101) or CRX06 ("I-DE"; DRB1*1102), but it does not, suggesting that it may be a new variant of DRw11, as discussed further below.

This sample, derived from an individual with bare lymphocyte syndrome, was 30 cloned and sequenced to determine the nature of the polymorphism at this position. Sequencing of the allele showed that there is indeed a sequence polymorphism at the third hypervariable region that codes for the epitope "F-DE"; this allele types as DRB1*1103 (see sequence "Beguit" in Table 3). Probe CRX68 hybridizes specifically to this polymorphism in the third hypervariable region (see Table 4).

All of the other samples also hybridize to the GH56 "YSTS" probe, so they 35 could be DR3, DRw11, or DRw6. Because these other samples do not hybridize to the "E" probe, it is likely they are DR3 or DRw6. However, the samples do not hybridize

to the probes CRX06 ("I-DE") or CRX23 ("A-H"), which recognize sequences present in three common DRw6 alleles, DRB1*1301, DRB1*1302, and DRB1*1401; this suggests that they are not DRw6, but DR3.

To confirm this, the samples were amplified with the DRB1 specific primers
5 GH46/CRX37 and probed with the SSOs for DR3, GH125 ("Y") and CRX50 ("K-GR"). About 100 ng of cell line genomic DNA were amplified with the DRB1 specific primers GH46/CRX37 as described in Example 1. Each filter containing amplified cell line DNA (samples A through F) was hybridized simultaneously with a control filter (samples 1 through 11) containing HTC DNAs amplified with the DRB1 specific
10 primers. All filters were prepared as described in Example 1. The results are shown in Figure 8.

Each pair of filters was hybridized the SSO probes shown. The samples are:
(1) No DNA control; (2) DR1 HTC "KAS9003"; (3) DR2 HTC "SCHU"; (4) DR3
15 HTC "QBL"; (5) DR4 HTC "BSM"; (6) DR5 (DRw11) HTC "SPOO1O"; (7) DRw6
HTC "OMW"; (8) DR7 HTC "MOU"; (9) DRw8 HTC "SPACH"; (10) DR9 HTC
"DKB"; (11) DRw10 HTC "SHY"; (A) No DNA control; (B) Raji; (C) 616; (D)
20 Beguit; (E) RM3; and (F) RS225. After the hybridization and wash steps, the bound probes were detected by enhanced chemiluminescence.

As Figure 8 shows, Raji, 616, RM3, and RS225 hybridize to both the "Y" and
20 "K-GR", which types them as DR3 (DRw17). In summary, Raji, RM3, and RS225 type as DRw10/DRw17. RM3 and RS225 are cell lines derived from Raji, so it is not surprising that they have the same DRB type. Cell line 616 types as DR1/DRw17, and Beguit types as DR7/DRw11 but, as noted above, the Beguit cell line contains the
25 DRB1*1103 allele, which has the "F-DE" epitope. Previously, most DRw11 typing cells were observed to contain DRB1 alleles with the "F-DR" epitope. Thus, the Beguit cell line contains an unusual DRB1 allele for the DRw11 type.

In addition to cell line samples, DNA from three different sources was amplified and typed. One source is purified genomic DNA from a set of unrelated, heterozygous individuals from the Center for Study of Human Polymorphism (CEPH, Paris, France; 30 samples 555 to 863). Another source was cDNA made from mRNA of cancer tissue from patients with bladder carcinoma (samples 2426, 2446, 2540, 2671, 2755). Normal human bladder cells do not express Class II molecules, but various carcinoma cells have been reported as expressing Class II molecules.

The remaining sample (PSW) is a buccal swab amplified for HLA DRB directly
35 without DNA purification. For complete DR typing, the samples were amplified with both the generic DRB primers GH46/50 and the DRB1 specific PCR primers GH46/CRX37. Filter strips containing DNA samples amplified with the general probes

were probed with the first panel of probes (Table 4), and filter strips containing DNA samples amplified specifically for DRB1 were probed with the second panel of probes (Table 5).

Comparing the pattern of hybridization with Table 3 and Table 8, one can
5 unambiguously derive the DRB-type of the samples. The results for the typing of the heterozygous samples are shown in Figure 9. In Figure 9, samples which hybridized to a probe are shown with a "+" symbol. Blank cells indicate samples which did not hybridize to a particular probe.

Samples 555 to 863 are pure genomic DNA provided by CEPH. Sample
10 "PSW" is a sample amplified directly from a buccal swab. This sample was heated for 5 minutes at 95°C in 200 µl of 5% Chelex (Singer-Sam *et al.*, 1989, Amplifications 3:11). About 50 µl of this solution were amplified directly in 200 µl of reaction volume, and generic DRB and DRB1 specific amplification reactions were conducted for the sample for 40 cycles.

15 Samples 2426, 2446, 2540, 2671, and 2755 were amplified from bladder carcinoma cDNA preparations. The bladder carcinoma samples 2426, 2446, 2540, 2671, and 2755 were not analyzed with the SSOs GH125, CRX50, CRX56, and GH54, because these probes require amplification with the DRB1 specific primers GH46/CRX37. Because CRX37 is derived from intron sequence, it cannot be used to
20 amplify cDNA. ND is not determined.

The buccal swab sample, PSW, is DR4 Dw14 (DRB1*0404) and DRw11.

These data show that the system is capable of typing heterozygous DNAs from a variety of sources, from standard purified genomic DNA, from cDNAs synthesized from RNA, and from unusual sources, such as a buccal swab or single hair.

25

Example 8

DRB Typing - Reverse Dot Blot Format

In this embodiment of the invention, the DRB probes are fixed to a membrane, and the amplified target DNA is hybridized to the membrane-bound probe. The set of typing probes is designed so that each probe will hybridize to a specific target sequence
30 at the same temperature and salt concentration (and stay hybridized under the same wash conditions) as all other probes in the set. The PCR primers used in the amplification are biotinylated, as described in the book PCR Protocols, incorporated herein by reference, so that any amplified DNA that hybridizes to the membrane-bound probes can be easily detected.

35 In one embodiment, detection is carried out by reacting streptavidin (SA)-conjugated horseradish peroxidase with any biotinylated, amplified DNA hybridized to

the membrane-bound probe. The HRP thus becomes bound, through the SA-biotin interaction, to the amplified DNA and can be used to generate a signal by a variety of well known means, such as the generation of a colored compound, e.g., by the oxidation of tetramethylbenzidine (see U.S. Patent No. 4,789,630, incorporated herein by reference).

5 Although the probes can be fixed to the membrane by any means, a preferred method involves "tailing" an oligonucleotide probe about 13 to 25 nucleotides in length with a much longer sequence of poly-dT. The resulting poly-dT "tail" can then be reacted with amine groups on the membrane to fix the probe covalently to the

10 membrane. This reaction can be facilitated by UV irradiation.

Terminal deoxyribonucleotidyl transferase (TdT, Ratliff Biochemicals; for the reactions below assume a concentration of about 120 Units/ μ l, which is 100 pmol/ μ l) can be used to create a poly-dT tail on a probe, although one can also synthesize the tailed probe on a commercially available DNA synthesizer. When one uses a DNA 15 synthesizer to make the tailed probe, however, one should place the tail on the 5' end of the probe, so that undesired premature chain termination occurs primarily in the tail region.

TdT reactions should be carried out in volume of about 100 μ l containing 1X TdT salts, 200 pmol of oligonucleotide, 800 μ M dTT, and 60 units of TdT. 10X TdT salts is 1,000 mM K-cacodylate, 10 mM CoCl₂, 2 mM dithiothreitol, 250 mM Tris-Cl, pH 7.6, and is prepared as described by Roychoudhury and Wu, Meth. Enzymol. 65:43-62, incorporated herein by reference. A 10X stock solution of 8 mM dTTP can be prepared (neutralized to pH 7 with NaOH) for convenience.

The TdT reaction should be carried out at 37°C for two hours and then stopped 25 by the addition of 100 μ l of 10 mM EDTA, pH 8. The final concentration of tailed oligonucleotide is 1 μ M (1 pmol/ μ l), and the length of the homopolymer tail is about 400 residues. Tail length can be changed by adjusting the molar ratio of dTTP to oligonucleotide. The tailed probes can be stored at -20°C until use.

Two types of nylon membrane are preferred for the reverse dot blot format:
30 Biodyne™ nylon membrane, 0.45 micron pore size, manufactured by Pall; and Biotrans™ nylon membrane, 0.45 micron pore size, manufactured by ICN. The probes can be spotted onto the membrane very conveniently with the Bio-Dot™ dot blot apparatus manufactured by BioRad. Each probe is spotted onto a unique, discrete location onto the membrane. About 5 to 10 picomoles of each tailed probe is premixed
35 with 60-100 μ l of TE buffer before application to the dot blot apparatus. After dot blotting, the membrane is briefly placed on absorbent paper to draw off excess liquid.

The membrane is then placed inside a UV light box, such as the Stratalinker™ light box manufactured by Stratagene, and exposed to 50 to 60 millijoules of flux to fix the tailed probe to the nylon membrane. After a brief rinse (for about 15 minutes in hybridization solution) to remove unbound probe, the membrane is then ready for

5 hybridization with biotinylated PCR product. One-half to one picomole (one-quarter to one-half of a typical, 100 µl PCR mixture) of PCR product is added to each probe panel for hybridization. About 50 µl of streptavidin-horseradish peroxidase (SA-HRP, commercially available from PECL; see the instruction manual for the AmpliTide™ DQα DNA Typing Kit, incorporated herein by reference) conjugate can be added at this

10 time for convenience, but better signals will result if a separate SA-HRP incubation and wash, at room temperature, is performed after the stringency wash.

Hybridization is typically carried out at 50°C for 30 minutes in a water bath and with hybridization buffer composed of 0.5% SDS and 3X to 5X SSPE, most commonly 4X. Stringency washing is carried out at 50°C for 15 minutes in a water

15 bath and with wash solution composed of 0.1% SDS and 1X SSPE. A post-wash of 1X PBS at room temperature for 30 minutes can enhance signal quality.

The biotinylated primers for the reverse dot blot method and other useful primers of the invention are shown below. Note, however, that one or both of the primers can be biotinylated in an amplification and that the primers can be used for

20 amplification with any detection format.

| <u>Primer</u> | <u>Seq. ID No.</u> | <u>Sequence</u> |
|---------------|--------------------|-----------------------------------|
| CRX11 | SEQ ID NO: 62 | 5'-TCTAGAAGTACTCTACGTCT-3' |
| CRX28 | SEQ ID NO: 67 | B-CCGGATCCTTCGTGTCACAGCACG-3' |
| CRX29 | SEQ ID NO: 68 | B-CTCCCCAACCCGTAGTTGTCTGCA-3' |
| 25 DRB17 | SEQ ID NO: 73 | B-GAATTCCCGCGCCGCCGCT-3' |
| DRB30 | SEQ ID NO: 107 | B-GAATTCCCGCGCCGCCGCTCACCT-3' |
| DRB152 | SEQ ID NO: 228 | B-CCCGTAGTTGTCTGCACACGG-3' |
| DB259 | SEQ ID NO: 95 | B-GAATTCCCGCGCCGCCGCTCACCTCG-3' |
| DB260 | SEQ ID NO: 96 | B-GAATTCCCGCGCCGCCGCTCACCTCGCC-3' |

30 B is biotin. CRX11 is a left-end primer designed for use with GH50 to amplify the DRB1 second exon of DR3, DR5, and DRw6 (13 and 14) haplotypes. CRX28 is biotinylated left-end primer GH46; CRX29 is biotinylated right-end primer GH50; and DRB17 is biotinylated right-end primer CRX37. DRB30 is a right-end primer that includes the CRX37 sequence and has the same DRB1 range as CRX37 except that,

35 unlike CRX37, DRB30 can amplify DR7 DRB1 sequences. DRB152 is a group specific right-end primer for DR7 and DR9 alleles. DB259 and DB260 are also right-

end primers designed to extend the range of DRB1 specific amplification to DR2 and DR9.

The hybridizing regions of the tailed probes for use in the reverse dot blot method are shown below. X is inosine. Where two probe names are shown, i.e.,

- 5 DRB01/CRX60, the first name designates the hybridizing region (shown) of the tailed probe, the second designates the HRP-labeled, untailed probe.

| <u>Primer</u> | <u>Seq. ID No.</u> | <u>Sequence</u> |
|----------------|--------------------|---------------------------|
| CRX23 | SEQ ID NO: 66 | S 5'-CCTGCTGCGGAGCACTG |
| GH54 | SEQ ID NO: 85 | U 5'-GCTGTTCCAGGACTC |
| 10 GH56 | SEQ ID NO: 86 | S 5'-CAGACGTAGAGTACTCC |
| GH59 | SEQ ID NO: 87 | U 5'-CATGTTAACCTGCTCC |
| GH102 | SEQ ID NO: 89 | S 5'-GAAATAACACTCACCCGTAG |
| GH104 | SEQ ID NO: 90 | S 5'-TGACACTCCCTCTTAGGCT |
| GH105 | SEQ ID NO: 91 | U 5'-CTTGCAGCAGGATAAGTATG |
| 15 GH111 | SEQ ID NO: 92 | S 5'-TTGAAGCAGGATAAGTTGA |
| GH122 | SEQ ID NO: 93 | S 5'-CAGTACTCCTCATCAGG |
| GH125 | SEQ ID NO: 94 | U 5'-CTGTCCAGGTACCGCAC |
| DRB01/CRX60 | SEQ ID NO: 79 | S 5'-CAAACCTAACGCTGCCAC |
| DRB02/CRX06 | SEQ ID NO: 61 | S 5'-CATCCTGGAAGACGAGC |
| 20 DRB03/CRX35 | SEQ ID NO: 71 | U 5'-CCTGTCTTCAGGAAGT |
| DRB04/CRX49 | SEQ ID NO: 74 | U 5'-TGACACTTATACTTACC |
| DRB05/CRX34 | SEQ ID NO: 70 | U 5'-CTCAAACCTAACCTCCTC |
| DRB06/CRX04 | SEQ ID NO: 60 | U 5'-GAGCAGAGGCGGGCC |
| DRB07/CRX68 | SEQ ID NO: 84 | S 5'-GACTCCTGGAAGACGA |
| 25 DRB08/CRX12 | SEQ ID NO: 63 | U 5'-AGCTGGGGCGGCCT |
| DRB09/CRX50 | SEQ ID NO: 75 | U 5'-CACCCGGCCCCGCTTCT |
| DRB10/CRX53 | SEQ ID NO: 76 | U 5'-GAGCAGAAGCGGGCC |
| DRB11/CRX15 | SEQ ID NO: 64 | U 5'-ACCTCGGCCCCGCTC |
| DRB12/CRX62 | SEQ ID NO: 81 | U 5'-ACATCCTGGAAGACAAG |
| 30 DRB13/CRX63 | SEQ ID NO: 82 | U 5'-ACATCCTGGAAGACAGG |
| DRB14/CRX61 | SEQ ID NO: 80 | U 5'-GCGGCCTAGCGCCGAGT |
| DRB15/CRX56 | SEQ ID NO: 77 | S 5'-CGGGTTGGTGAGAGCT |
| DRB16/CRX57 | SEQ ID NO: 78 | S 5'-CGGGTTGTGGAGAGCT |
| DRB19 | SEQ ID NO: 97 | S 5'-TGACACTTATACTTACCGTC |
| 35 DRB20 | SEQ ID NO: 98 | S 5'-CTCAAACCTAACCTCCTCC |
| DRB21 | SEQ ID NO: 99 | S 5'-GAGCAGAGGCGGGC |
| DRB22 | SEQ ID NO: 100 | U 5'-GCCTGTCTTCAGGAAGT |

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|----|-------|----------------|---|--------------------------|
| | DRB23 | SEQ ID NO: 101 | U | 5'-GCCCGCTTCTGCTC |
| | DRB24 | SEQ ID NO: 102 | U | 5'-GAGCAGAAGCXGGCC |
| | DRB25 | SEQ ID NO: 103 | U | 5'-CATCCTGGAAGACAGG |
| | DRB26 | SEQ ID NO: 104 | U | 5'-CATCCTGGAAGACAGGCG |
| 5 | DRB27 | SEQ ID NO: 105 | S | 5'-ACATCCTGGAAGACAAGC |
| | DRB28 | SEQ ID NO: 107 | U | 5'-ACCTCGGCCXCCTC |
| | DRB31 | SEQ ID NO: 108 | S | 5'-CTCCCGTTATGGATGTATC |
| | DRB32 | SEQ ID NO: 109 | S | 5'-TGTCCAGGTACCGCA |
| | DRB33 | SEQ ID NO: 110 | S | 5'-ACTCATGTTAACCTGCTCC |
| 10 | DRB34 | SEQ ID NO: 111 | S | 5'-CTCATGTTAACCTGCTCC |
| | DRB35 | SEQ ID NO: 112 | S | 5'-TGTCCCGAGTCCTGG |
| | DRB36 | SEQ ID NO: 113 | S | 5'-CGCCTGTCITCCAGGAAGT |
| | DRB37 | SEQ ID NO: 114 | S | 5'-CCGCCTGTCTCCAGGAAGT |
| | DRB38 | SEQ ID NO: 115 | U | 5'-TCCACCCGGCCCCGTTCT |
| 15 | DRB39 | SEQ ID NO: 116 | U | 5'-GAGCAGAAGCGGGC |
| | DRB40 | SEQ ID NO: 117 | U | 5'-GAGXAGAAGCXGGCC |
| | DRB41 | SEQ ID NO: 118 | U | 5'-GGCCCGCTTCTGCTC |
| | DRB42 | SEQ ID NO: 119 | S | 5'-GACGGAGCTGGGGCGGC |
| | DRB43 | SEQ ID NO: 120 | U | 5'-GACTCCTGGAGCGGAGG |
| 20 | DRB44 | SEQ ID NO: 121 | U | 5'-GACCTCCTGGAGCGGAGG |
| | DRB45 | SEQ ID NO: 122 | S | 5'-GAGCGGAGGCCTGCC |
| | DRB46 | SEQ ID NO: 123 | S | 5'-TCAGACGTAGAGTACTCC |
| | DRB47 | SEQ ID NO: 124 | U | 5'-TCTTGCAGCAGGATAAGTATG |
| | DRB48 | SEQ ID NO: 125 | S | 5'-CACTCATGTTAACCTGCTCC |
| 25 | DRB49 | SEQ ID NO: 126 | U | 5'-TCAGACTTACCGCAGCTCC |
| | DRB50 | SEQ ID NO: 127 | U | 5'-TCAGACTTAAGCAGCTCC |
| | DRB51 | SEQ ID NO: 128 | U | 5'-GAGCAGAGGCGGAGC |
| | DRB52 | SEQ ID NO: 129 | U | 5'-GAGCAGAAGCGAGGCC |
| | DRB53 | SEQ ID NO: 130 | U | 5'-GGCCCGCTTCTGCTCCA |
| 30 | DRB54 | SEQ ID NO: 131 | U | 5'-GCGGCCCGCTTCTGCTC |
| | DRB55 | SEQ ID NO: 132 | U | 5'-GAGCAGAAGCGAGGC |
| | DRB56 | SEQ ID NO: 133 | U | 5'-GGAGXAGAAGCXGGCCG |
| | DRB57 | SEQ ID NO: 134 | U | 5'-CCACCCGGCCCCGTTCT |
| | DRB58 | SEQ ID NO: 135 | U | 5'-CATCCTGGAAGACAGGCG |
| 35 | DRB59 | SEQ ID NO: 136 | U | 5'-CATCCTGGAAGACAGAGCG |
| | DRB60 | SEQ ID NO: 137 | S | 5'-GGCGGCCTAGCGCCGAGT |
| | DRB61 | SEQ ID NO: 138 | U | 5'-ACCTCCTGGAGCGGAGG |

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|----|-------|----------------|---|----------------------------|
| | DRB62 | SEQ ID NO: 139 | S | 5'-GACTTCCTGGAGCGGAG |
| | DRB63 | SEQ ID NO: 140 | S | 5'-CATCCTGGAGCAGGCG |
| | DRB64 | SEQ ID NO: 141 | U | 5'-ACCTCGGCCXCCCTCTG |
| | DRB66 | SEQ ID NO: 142 | U | 5'-GAGCAGAAGCGGG |
| 5 | DRB67 | SEQ ID NO: 143 | U | 5'-GAGXAGAAGCXGGCCG |
| | DRB68 | SEQ ID NO: 144 | U | 5'-CCTCGGCCXCCCTCTGC |
| | DRB69 | SEQ ID NO: 145 | U | 5'-CCTCCTGGAGCGGAGG |
| | DRB70 | SEQ ID NO: 146 | U | 5'-CGCCTGTCTTCCAGGATG |
| | DRB71 | SEQ ID NO: 147 | U | 5'-TTCTTGCAAGCAGGATAAGTATG |
| 10 | DRB72 | SEQ ID NO: 148 | S | 5'-CGCCTGTCTCCAGGATG |
| | DRB73 | SEQ ID NO: 149 | U | 5'-AGAACGGGGCCGGGTG |
| | DRB74 | SEQ ID NO: 150 | U | 5'-GAGCAGAGXCAGGCC |
| | DRB75 | SEQ ID NO: 151 | U | 5'-GAGCAGAGAGCGGGC |
| | DRB76 | SEQ ID NO: 152 | U | 5'-CTTCTGCTCCAGGAGG |
| 15 | DRB77 | SEQ ID NO: 153 | U | 5'-CTCCTGGAGCAGAAG |
| | DRB78 | SEQ ID NO: 154 | U | 5'-CCTCCTGGAGCXGAAG |
| | DRB79 | SEQ ID NO: 155 | U | 5'-CCTCCTGGAGCAAGAAG |
| | DRB80 | SEQ ID NO: 156 | U | 5'-CGGCCGCCCTCTGCTC |
| | DRB81 | SEQ ID NO: 157 | U | 5'-CGGGGCTGTGGAGAGCT |
| 20 | DRB82 | SEQ ID NO: 158 | U | 5'-CTTCTGCTCCAGGAGGTC |
| | DRB83 | SEQ ID NO: 159 | U | 5'-ACCTCCTGGAGCAGAAG |
| | DRB84 | SEQ ID NO: 160 | S | 5'-GGCCCGCCTCTGCTC |
| | DRB85 | SEQ ID NO: 161 | U | 5'-GCCCGCCTCTGCTC |
| | DRB86 | SEQ ID NO: 162 | U | 5'-GGCCCGCCTCTGC |
| 25 | DRB87 | SEQ ID NO: 163 | U | 5'-GAGGCGCGCCGAGGT |
| | DRB88 | SEQ ID NO: 164 | U | 5'-GAGGCGCGCCGAGGTG |
| | DRB89 | SEQ ID NO: 165 | U | 5'-GAGGCGCGCCGAGGTGGA |
| | DRB90 | SEQ ID NO: 166 | S | 5'-AGAACGGGGCCGGGT |
| | DRB91 | SEQ ID NO: 167 | S | 5'-AGAACGGGGCCGGG |
| 30 | DRB92 | SEQ ID NO: 168 | U | 5'-GGGGTTGGTGAGAGCT |
| | DRB93 | SEQ ID NO: 169 | U | 5'-GGGGTTGTGGAGAGCT |
| | DRB94 | SEQ ID NO: 170 | U | 5'-ACCTCGGCCGCCCTC |
| | DRB95 | SEQ ID NO: 171 | S | 5'-CATCCTGGAAGACAGGC |
| | DRB96 | SEQ ID NO: 172 | U | 5'-GAGCAGAAGCAGGCC |
| 35 | DRB97 | SEQ ID NO: 173 | U | 5'-GGCGGCCTAGCGCCGAGTAC |
| | DRB98 | SEQ ID NO: 174 | U | 5'-TGTAGGACCTCTGTCCG |
| | DRB99 | SEQ ID NO: 175 | U | 5'-GTAGGACCTCTGTCCG |

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|----|--------|----------------|---|-----------------------------|
| | DRB100 | SEQ ID NO: 176 | S | 5'-TTCTTGCAGCAGGATAAGTATGAG |
| | DRB101 | SEQ ID NO: 177 | S | 5'-CTCCCGTTATGGATGTATC |
| | DRB102 | SEQ ID NO: 178 | S | 5'-CAGTACTCCTCATCAGGC |
| | DRB103 | SEQ ID NO: 179 | S | 5'-CTGTCCAGGTACCGCA |
| 5 | DRB104 | SEQ ID NO: 180 | U | 5'-ACCTCGGCCGCCCTCT |
| | DRB105 | SEQ ID NO: 181 | U | 5'-GAGGCGCGCCGAGGTGGAC |
| | DRB106 | SEQ ID NO: 182 | U | 5'-AGAGGCGCGCCGAGGTGGAC |
| | DRB107 | SEQ ID NO: 183 | S | 5'-AGAACGGGGCCGG |
| | DRB108 | SEQ ID NO: 184 | S | 5'-GAAGCGGGGCCGGG |
| 10 | DRB109 | SEQ ID NO: 185 | S | 5'-GAAGACAGGCGGGCCCTGG |
| | DRB110 | SEQ ID NO: 186 | U | 5'-GCCTGTCITCCAGGAAGTCC |
| | DRB111 | SEQ ID NO: 187 | U | 5'-CTCAGACGTAGAGTACTCC |
| | DRB112 | SEQ ID NO: 188 | S | 5'-GCCTGCTGCGGAGCACTGG |
| | DRB113 | SEQ ID NO: 189 | S | 5'-GACCTCCTGGAAGACAGG |
| 15 | DRB114 | SEQ ID NO: 190 | U | 5'-CCTGTCCTCCAGGAGGTC |
| | DRB115 | SEQ ID NO: 191 | U | 5'-ACGGGGTTGGTGAGAGCTT |
| | DRB116 | SEQ ID NO: 192 | U | 5'-ACGGGGTTGTGGAGAGCTT |
| | DRB117 | SEQ ID NO: 193 | U | 5'-ACGGGGCTGTGGAGAGCTT |
| | DRB118 | SEQ ID NO: 194 | S | 5'-GAGGCGGGCCGAGGT |
| 20 | DRB119 | SEQ ID NO: 195 | U | 5'-GAGGCGGGCCGAGGTG |
| | DRB120 | SEQ ID NO: 196 | U | 5'-GAGGCGGGCCGAGGTGGA |
| | DRB121 | SEQ ID NO: 197 | U | 5'-GAGGCGGGCCGAGGTGGAC |
| | DRB122 | SEQ ID NO: 198 | U | 5'-AGAGGCGGGCCGAGGTGGAC |
| | DRB123 | SEQ ID NO: 199 | T | 5'-GGCGGCCTAGCGCCGAGTA |
| 25 | DRB124 | SEQ ID NO: 200 | T | 5'-CCACXCGGCCCGCTTCT |
| | DRB125 | SEQ ID NO: 201 | T | 5'-GAGGCGGGCCCGCGGT |
| | DRB126 | SEQ ID NO: 202 | T | 5'-ACCGCGGCCGCCCTC |
| | DRB127 | SEQ ID NO: 203 | T | 5'-GAAGCGGGCCGCGGT |
| | DRB128 | SEQ ID NO: 204 | T | 5'-ACCGCGGCCGCCCTTC |
| 30 | DRB129 | SEQ ID NO: 205 | T | 5'-ACTCCTGGAAGACAGG |
| | DRB130 | SEQ ID NO: 206 | T | 5'-CGCAAGTCCTCCTCTTG |
| | DRB131 | SEQ ID NO: 207 | T | 5'-CAAGAGGAGGACTTGCG |
| | DRB132 | SEQ ID NO: 208 | T | 5'-GAAGACAGGCGGGCCCTG |
| | DRB133 | SEQ ID NO: 209 | T | 5'-AAGACAGGCGGGCCCTGG |
| 35 | DRB134 | SEQ ID NO: 210 | T | 5'-ACTCCTGGAAGACGAG |
| | DRB135 | SEQ ID NO: 211 | T | 5'-ACTCCTGGAAGACGAGC |
| | DRB136 | SEQ ID NO: 212 | T | 5'-ACATCCTGGAAGACAGGC |

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|----|--------|----------------|-----------------------------|
| | DRB137 | SEQ ID NO: 213 | T 5'-GCCTGTCTTCCAGGATG |
| | DRB138 | SEQ ID NO: 214 | T 5'-GCAGAAGCGGGCCGCG |
| | DRB139 | SEQ ID NO: 215 | T 5'-CGCGGCCCGCTCTGC |
| | DRB140 | SEQ ID NO: 216 | T 5'-GCAGAGGCAGGGCCGCG |
| 5 | DRB141 | SEQ ID NO: 217 | T 5'-CGCGGCCCGCCTCTGC |
| | DRB142 | SEQ ID NO: 218 | T 5'-GCAGAGGCAGGGCCGAG |
| | DRB143 | SEQ ID NO: 219 | T 5'-CTCGGCCCGCCTCTGC |
| | DRB144 | SEQ ID NO: 220 | T 5'-GCAGAGGCAGGGCCGAG |
| | DRB145 | SEQ ID NO: 221 | T 5'-CTCGGCCCGCCTCCGC |
| 10 | DRB146 | SEQ ID NO: 222 | T 5'-CTCCGCTCCAGGAAGTC |
| | DRB147 | SEQ ID NO: 223 | T 5'-CGGGGTTGGTGAGAGCT |
| | DRB148 | SEQ ID NO: 224 | T 5'-CGGGGTTGTGGAGAGCT |
| | DRB149 | SEQ ID NO: 225 | T 5'-CGGGGCTGTGGAGAGCTT |
| | DRB150 | SEQ ID NO: 226 | 5'-TGTCCACCGCGGCCCCGCGCCT |
| 15 | DRB153 | SEQ ID NO: 229 | 5'-GAATTCCCAGCTCACACGGGACT |
| | DRB154 | SEQ ID NO: 230 | 5'-GGTGTCCACCGCGGCCCCGCGC |
| | DRB155 | SEQ ID NO: 231 | 5'-AACCCCGTAGTTGTGTCTGCACAC |
| | DRB156 | SEQ ID NO: 232 | 5'-GGGGGAGTTCCGGG |
| | DRB157 | SEQ ID NO: 233 | 5'-CCCGGTACTCCCC |
| 20 | DRB158 | SEQ ID NO: 234 | 5'-CGCGGCCCGCCTCTG |
| | DRB159 | SEQ ID NO: 235 | 5'-CCGCGGCCCGCCTCTG |
| | DRB160 | SEQ ID NO: 236 | 5'-CCGXGGCCCCGCTCTGC |
| | DRB161 | SEQ ID NO: 237 | 5'-CCAGCGGCCCGCCTCTGC |
| | DRB162 | SEQ ID NO: 238 | 5'-GCAGAACXGGGCCGXGT |
| 25 | DRB163 | SEQ ID NO: 239 | 5'-GCAGAAAGCGGGCCGXGT |
| | DRB164 | SEQ ID NO: 240 | 5'-CAGAACGGGCCGCG |
| | DRB165 | SEQ ID NO: 241 | 5'-ACCTXGGCCCCGCCXCTGC |
| | DRB166 | SEQ ID NO: 242 | 5'-ACCTXGGCCCCGCCXCTG |
| | DRB167 | SEQ ID NO: 243 | 5'-GGAGCAGAAACGGGCCG |
| 30 | DRB168 | SEQ ID NO: 244 | 5'-GGAGCAGAAACGGGCCGC |
| | DRB169 | SEQ ID NO: 245 | 5'-GCAGAACGGGCCXCG |
| | DRB170 | SEQ ID NO: 246 | 5'-GTCCACCTCGGCCCG |
| | DRB171 | SEQ ID NO: 247 | 5'-CGGGCCGCGGTGGAC |
| | DRB172 | SEQ ID NO: 248 | 5'-CGCCTCCGCTCCAGGAG |
| 35 | DRB173 | SEQ ID NO: 249 | 5'-CTCCTGGAGCAGAGGCG |
| | DRB174 | SEQ ID NO: 250 | 5'-ACCAGCGGCCGCCCT |
| | DRB175 | SEQ ID NO: 251 | 5'-CACCTXGGCCCCGCCXCTG |

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|----|--------|----------------|--------------------------|
| | DRB176 | SEQ ID NO: 252 | 5'-CGXGGCCCGCCTCTG |
| | DRB177 | SEQ ID NO: 253 | 5'-CCGXGGCCCGCCTCTG |
| | DRB178 | SEQ ID NO: 254 | 5'-GGGGGAGTTCCGGGCG |
| | DRB179 | SEQ ID NO: 255 | 5'-CGCCCGGTACTCCCC |
| 5 | DRB180 | SEQ ID NO: 256 | 5'-XCCTGATGCCGAGTACTG |
| | DRB181 | SEQ ID NO: 257 | 5'-XCGGGGCTGTGGAGAGCTT |
| | DRB182 | SEQ ID NO: 258 | 5'-CTACGGGGCTGTGGAGAG |
| | DRB183 | SEQ ID NO: 259 | 5'-CTACGGGXCTGTGGAGAG |
| | DRB184 | SEQ ID NO: 260 | 5'-GTTCCGGCGGGTGAC |
| | DRB185 | SEQ ID NO: 261 | 5'-GGGGGAGTTXCGGGG |
| 10 | DRB186 | SEQ ID NO: 262 | 5'-CGTCACCGCCCGGTAC |
| | DRB187 | SEQ ID NO: 263 | 5'-CGTCACCGCCCGXTAC |
| | DRB188 | SEQ ID NO: 264 | 5'-CACCCCTCATXGCC |
| | DRB189 | SEQ ID NO: 265 | 5'-GCAGGGCCGCGGTGGAC |
| | DRB190 | SEQ ID NO: 266 | 5'-CAGAGGCXGGCCGCGGT |
| 15 | DRB191 | SEQ ID NO: 267 | 5'-GTTXCGGGCGGTGAC |
| | DRB192 | SEQ ID NO: 268 | 5'-TTCCGGGCGGTGAC |
| | DRB193 | SEQ ID NO: 269 | 5'-GGGGAGTTCCGGG |
| | DRB194 | SEQ ID NO: 270 | 5'-TCACCGCCCCGAAC |
| | DRB195 | SEQ ID NO: 271 | 5'-AGATACTTCTATAACCAG |
| 20 | DRB196 | SEQ ID NO: 272 | 5'-AGACACTTCTATAACCAG |
| | DRB197 | SEQ ID NO: 273 | 5'-CTGGTTATAGAAGTATCT |
| | DRB198 | SEQ ID NO: 274 | 5'-CTGTCGCCGAGTCCTGG |
| | DRB199 | SEQ ID NO: 275 | 5'-GGGCGGCCTAGCGCCGAGT |
| | DRB200 | SEQ ID NO: 276 | 5'-GCAGAAAGCGGGCCGCXGT |
| 25 | DRB201 | SEQ ID NO: 277 | 5'-GCGXCTGTCTCCAGGATG |
| | DRB202 | SEQ ID NO: 278 | 5'-CGXCTGTCTCCAGGATG |
| | DRB203 | SEQ ID NO: 279 | 5'-ACCGXGGCCCGCCTCTG |
| | DRB204 | SEQ ID NO: 280 | 5'-CCGTCACCGCCCGXTAC |
| | DRB205 | SEQ ID NO: 281 | 5'-GGGGAGTTCCGGGG |
| 30 | DRB206 | SEQ ID NO: 282 | 5'-TCACCGCCCCGAACTC |
| | DRB207 | SEQ ID NO: 283 | 5'-TGACACTTATACTTACCTGC |
| | DRB208 | SEQ ID NO: 284 | 5'-TTGAAGCAGGATAAGTTGAG |
| | DRB209 | SEQ ID NO: 285 | 5'-CTTGAAGCAGGATAAGTTG |
| | DRB210 | SEQ ID NO: 286 | 5'-GAATTCCCGCGCCGCGCTCA |
| 35 | DRB211 | SEQ ID NO: 287 | 5'-GAATTCCCGCGCCGCG |
| | DRB212 | SEQ ID NO: 288 | 5'-GAATTCCCGCGCCGCGCTCAC |

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|----|--------|----------------|-----------------------------|
| | DRB213 | SEQ ID NO: 289 | 5'-ATGACACTCCCTCTTAGGCTG |
| | DRB214 | SEQ ID NO: 290 | 5'-ACATCCTGGAAGACGAG |
| | DRB215 | SEQ ID NO: 291 | 5'-CCGCTCCGTCCCATTGAA |
| | DRB216 | SEQ ID NO: 292 | 5'-TTCAATGAGACGGAGCGG |
| 5 | DRB217 | SEQ ID NO: 293 | 5'-CATCCTGGAAGACGAG |
| | DRB218 | SEQ ID NO: 294 | 5'-GCTCGTCTTCAGCATG |
| | DRB219 | SEQ ID NO: 295 | 5'-CGCTCGTCTTCAGGATG |
| | DRB220 | SEQ ID NO: 296 | 5'-GCTGTCGCCGAGTCCTGG |
| | DRB221 | SEQ ID NO: 297 | 5'-CCTGTCGCCGAGTCCTGG |
| 10 | DRB222 | SEQ ID NO: 298 | 5'-CTGTCAGGTACCGCA |
| | DRB223 | SEQ ID NO: 299 | 5'-GGCGGCCTAGCGCCGAGTA |
| | DRB224 | SEQ ID NO: 300 | 5'-CCACXCGGCCCGCTTCT |
| | DRB225 | SEQ ID NO: 301 | 5'-GAGGCGGGCCGCGGT |
| | DRB226 | SEQ ID NO: 302 | 5'-ACCGCGGCCCGCCTC |
| 15 | DRB227 | SEQ ID NO: 303 | 5'-GAAGCGGGCCGCGGT |
| | DRB228 | SEQ ID NO: 304 | 5'-ACCGCGGCCCGCTTC |
| | DRB229 | SEQ ID NO: 305 | 5'-ACTTCCTGGAAGACAGG |
| | DRB230 | SEQ ID NO: 306 | 5'-GACCTCCTGGAAGACAGG |
| | DRB231 | SEQ ID NO: 307 | 5'-ACATCCTGGAAGACAAGC |
| 20 | DRB232 | SEQ ID NO: 308 | 5'-GACATCCTGGAAGACAAGC |
| | DRB233 | SEQ ID NO: 309 | 5'-ACATCCTGGAAGACAAGCG |
| | DRB300 | SEQ ID NO: 310 | 5'-GAATTCCCGCGCCGCGCTCACCTC |
| | DRB305 | SEQ ID NO: 311 | 5'-GAATTACAGGGACTCCAGGCC |

Preliminary testing shows that some probes, marked "S" above are more preferred than other probes, marked "U" above. Other probes, marked "T" above, have not yet been tested.

The hybridization patterns and specificity (1989 allele set) for the preferred reverse dot blot probes are show below. Where an "X" is used in the "specificity" column, the "X" designates inclusivity of all alleles indicated by the first two digits after the "*".

| <u>Epitope</u> | <u>Name</u> | <u>Specificity</u> |
|----------------|--------------|--|
| W-L-F | CRX60/DRB01 | DRB1*0101, 0102, 0103 |
| W-P-R | GH104 | DRB1*1501, 1502, 1601, 1602 |
| QDY | DRB100 | DRB5*0101, 0102, 0201, 0202 |
| 5 K-D-F | GH111 | DRB1*0901 |
| YSTS | DRB46 | DRB1*030X, 110X, 130X, 140X |
| YSTG | GH102 | DRB1*080X, 1201 |
| V-H | DRB48 | DRB1*040X |
| G-YK | DRB19 | DRB1*070X |
| 10 EV | DRB20 | DRB1*1001 |
| LR-S | GH57 | DRB3*0101 |
| LL-S | GH58 | DRB3*0201, 0202, 0301 |
| F-DR | DRB37/DRB109 | DRB1*0801, 0802, 1101, 1104, 1601, PEV; DRB5*0101, 0102 |
| 15 F-DE | CRX68 | DRB1*1103 |
| I-DE | CRXO6/DRB02 | DRB1*0103, 0402, 1102, 1301, 1302 |
| I-DK | DRB27 | DRB1*1303 |
| RR | DRB45 | DRB1*1001 |
| F--RR-E | DRB62 | DRB1*0901 |
| 20 I-A | DRB63 | DRB1*1501, 1502; DRB5*0201, 0202 |
| Y | DRB103 | DRB1*0301, DRB3*0101 |
| E | DRB102 | DRB1*110X |
| S | DRB60 | DRB1*0405, 0801, 0803, 1303 |
| A--H | DRB112 | DRB1*1401, LY10 |
| 25 V-S | DRB35 | DRB1*070X, 0901, 1201; DRB3*0101, 0301 |
| R | --- | DRB1*0101, 0102, 0404, 0405, 0408, 1402 |
| K | --- | DRB1*0401 |
| 30 R-E | --- | DRB1*0403, 0406, 0407 |
| RR-E | --- | DRB1*1401, LY10; DRB4*0101 |
| I-DR | DRB72 | DRB1*0701, 0702 |
| I-DR | DRB95 | DRB1*0803, 1201 |
| K-GR | --- | DRB1*0301, 0302; DRB3*0101 |
| 35 DR | DRB113 | DRB1*1602 |
| G (pos. 86) | --- | See Table 5 |
| V (pos. 86) | --- | See Table 5 |

| <u>Epitope</u> | <u>Name</u> | <u>Specificity</u> |
|----------------|-------------|-----------------------|
| AV (pos. 86) | --- | DRB1*0102, 1201; DRB5 |
| WNIIN | GH51 | DRB4*0101 |
| IHKR | DRB101 | DRB2*0101 |

5

Example 9Reverse Dot-Blot Typing Kit

- HLA DRB typing kits incorporating a rapid and simple reverse dot blot hybridization format were designed to provide a simple and fast prescreening of samples before proceeding to fine subtyping involving allele specific amplification.
- 10 The kits contain the amplification reagents, DNA for use as a positive control, nylon strips on which the probes have been immobilized, colorimetric detection reagents, reaction tubes, and instructions.

Typing was carried out using two amplification reactions, one using DRB general primers and the other using DRB1 specific primers. The primer pairs amplify under the same thermocycler conditions so that the two reactions could be carried out concurrently. Two panels of probes, one specific for each amplification reaction, were used in the reverse dot blot hybridization format. Each panel of probes was immobilized on a single nylon strip for ease of handling.

Biotinylated primers were used in the amplification reactions to allow for later detection using a colorimetric assay as described in Example 8, above. The DRB amplification primers used were CRX28 (SEQ ID NO: 67) and CRX29 (SEQ ID NO: 68). The DRB1 amplification primers were CRX28 and CRX 37 (SEQ ID NO: 73). Both sets of primers are described in Example 8, above.

The two probe panels, one for hybridizing with the amplification products of the DRB amplification and the other for hybridizing with the amplification product from the DRB1-specific amplification, are shown below. The nucleotide sequence for each probe is found in the Sequence Listing section; the SEQ ID NO: for each probe is provided below.

Probe Panel for the DRB Amplification

| | <u>Probe</u> | <u>Seq. ID No.</u> | <u>AA Sequence</u> | <u>Reactivity</u> |
|----|--------------|--------------------|--------------------|-------------------|
| 1 | DRB01 | SEQ ID NO: 79 | WLF | DR1 |
| 2 | GH104 | SEQ ID NO: 90 | WPR | DR2 |
| 5 | 3 DRB46 | SEQ ID NO: 123 | YSTS | DR3, 11, 13, 14 |
| | 4 DRB48 | SEQ ID NO: 125 | V-H | DR4 |
| | 5 DRB207 | SEQ ID NO: 283 | G-YK | DR7 |
| | 6 GH102 | SEQ ID NO: 89 | YSTG | DR8, 12, 1404 |
| | 7 DRB209 | SEQ ID NO: 285 | K-D-F | DR9 |
| 10 | 8 DRB20 | SEQ ID NO: 98 | EV | DR10 |
| | 9 DRB102 | SEQ ID NO: 178 | E | DR11 |
| | 10 DRB112 | SEQ ID NO: 188 | A-H | 1401, 1404 |
| | 11 DRB07 | SEQ ID NO: 84 | F-DE | 1103 |
| | C DRB42 | SEQ ID NO: 119 | TELGRP | ALL |

Probe Panel for the DRB1 Amplification

| | <u>Probe</u> | <u>Seq. ID No.</u> | <u>AA Sequence</u> | <u>Reactivity</u> |
|----|--------------|--------------------|--------------------|----------------------|
| 12 | DRB223 | SEQ ID NO: 299 | S | * |
| 13 | DRB37 | SEQ ID NO: 114 | F-DR | * |
| 14 | DRB203 | SEQ ID NO: 279 | R | * |
| 20 | 15 DRB163 | SEQ ID NO: 239 | K | 0401, 0409 |
| | 16 DRB118 | SEQ ID NO: 194 | R-E/RR-E | * |
| | 17 DRB02 | SEQ ID NO: 61 | I-DE | * |
| | 18 DRB38 | SEQ ID NO: 115 | K-GR | DR3 |
| | 19 DRB222 | SEQ ID NO: 298 | Y | 0301 |
| 25 | 20 DRB232 | SEQ ID NO: 308 | I-DK | 1303 |
| | 21 DRB136 | SEQ ID NO: 212 | I-DR | 0803, 1201 (not DR7) |
| | 22 DRB198 | SEQ ID NO: 274 | V-S | DR7, 0803, 1201 |
| | C DRB42 | SEQ ID NO: 119 | TELGRP | ALL |

* sequence is found on a number of different alleles (see DRB1 amino acid alignment).

30 DRB probes 1-8 are specific to the region about amino acids 9-13; probe 9 is specific for amino acid 58; probe 10 is specific for amino acids 57-60; and probe 11 is specific for amino acids 67-74. DRB1 probes 13-18, 20, and 21 are specific to amino acids 67-74; probe 12 is specific to amino acid 57; probe 20 is specific to amino acid 26; and probe 22 is specific for amino acids 57-60. The control probe is specific for 35 amino acids 51-56.

It should be noted that the probes shown in the above panels can also be labeled and used in the DRB typing methods described in Example 7. The same hybridization and wash conditions would be used. Interpretation of the hybridization pattern would be as described below.

5 The kits were packaged as two boxes: one box contained the DRB reagents and the other box contained the DRB1 reagents. Packaged in each box was either DRB or DRB1 PCR mix, DNA control, and typing strips; 8 mM magnesium chloride solution; mineral oil; SA-HRP conjugate; chromogen (TMB); reaction tubes; and instructions. PCR contains the reagents necessary for a PCR with the exception of the magnesium 10 chloride and the template DNA. Other reagents and equipment needed to perform the methods described were supplied by the kit user; all are commonly available commercially.

Several sample preparation procedures suitable for use in PCR amplifications are known in the art. A preferred procedure is the Chelex extraction method described 15 in Singer-Sam *et al.*, 1989, Amplifications 3:11, and Walsh *et al.*, 1991, BioTechniques 10(4):506-513, both of which are incorporated herein by reference. For examples of other techniques for extracting nucleic acids from biological samples, see those described in Sambrook *et al.*, Molecular Cloning: A Laboratory Manual (New York, Cold Spring Harbor Laboratory, 1989); Arrand, Preparation of Nucleic 20 Acid Probes, in pp. 18-30, Nucleic Acid Hybridization: A Practical Approach (Ed Hames and Higgins, IRL Press, 1985); or, in PCR Protocols, Chapters 18-20 (Innis *et al.*, ed., Academic Press, 1990), which are all incorporated herein by reference.

Primer pairs for amplifying all DRB sequences and for amplifying specifically DRB1 sequences are used in two separate PCR reactions. Amplification reactions are 25 essentially as described in Saiki *et al.*, 1989, Proc. Natl. Acad. Sci. USA 86:6230-6234, incorporated herein by reference, with the modifications described below. Reactions are carried out using 25 µl of sample in a total volume of 100 µl, each reaction containing 50 mM KCl, 10 mM TrisHCl (pH 8.4), 1.5 mM MgCl₂, 10 µg Gelatin, 200 µM each dATP, dCTP, dGTP, and dTTP, 0.2 µM each biotinylated 30 amplification primer, and 2.5 units of *Thermus aquaticus* DNA polymerase (PECI).

The same temperature profile is used for both amplification reaction, allowing both reaction to be carried out concurrently in the same thermocycler. The thermocycler is programmed for 35 cycles of the following temperature profile: denature at 95°C for 60 seconds, anneal at 60°C for 30 seconds, extend at 72°C for 60 seconds. The 35 thermocycler is programmed to incubate the sample for an additional 7 minutes at 72°C following the last cycle.

The amplified DNA from the PCR is in double stranded form and must be denatured to allow hybridization with the oligonucleotide probes. The amplified DNA is denatured at 95°C for 5 to 10 minutes in the thermocycler and maintained at that temperature until used. Alternatively, amplified DNA which has been denatured can be 5 transferred directly from the 95°C temperature into an ice bath. The rapid cooling stabilizes the DNA in a denatured form. The amplified DNA is then kept in the ice bath until it is required for the hybridization.

Twelve probes are affixed to each of two nylon membrane strips; one strip contains the probes to be used for hybridization with the DRB amplification product 10 and the other strip contains the probes for hybridization with the DRB1-specific amplification product. Hybridization reactions are carried out in trays which hold each probe-containing strip in a separate well. The hybridization trays are commercially available from PECL and are typically supplied with the kit. The hybridization conditions described below are specific for the DRB hybridization. The DRB1 DNA 15 hybridization protocol differs from the DRB protocol in that the DRB1 hybridization is at 55°C instead of the 50°C. All other aspects of the protocol are identical.

In each well containing a strip, 3 ml of pre-warmed hybridization solution (4X SSPE and 0.5% weight/volume SDS) are added followed by 25 µl of amplified DNA. After careful mixing of the tray contents, the tray is placed in a 50°C shaking water 20 bath and incubated at 50°C for 20 minutes at about 50 rpm.

The strips are washed initially in 10 ml of wash solution (1.0X SSPE and 0.1% weight/volume SDS) at room temperature for several seconds prior to the stringent wash and each well is then aspirated. The temperature and timing of the stringent wash are critical. Pre-warmed wash solution (10 ml) are added to each well and the tray is 25 incubated in a 50°C shaking water bath for 12 (\pm 2) minutes at about 50 rpm. After aspirating each well, 10 ml of wash solution are added to each well, the tray is incubated at room temperature for 5 minutes on an orbital shaker at about 50 rpm, and the wells are again aspirated.

To each well containing a strip are added 3 ml of wash/enzyme conjugate 30 solution (from a solution of 3.3 ml of wash solution and 27 µl of enzyme conjugate prepared within 15 minutes of use). The tray is incubated at room temperature for 20 minutes on an orbital shaker at approximately 50 rpm. The solution is aspirated from each well and the strips are washed in 10 ml of wash solution at room temperature for 5 minutes on an orbital shaker at approximately 50 rpm. Finally, the wash solution is 35 aspirated from each well and the strips are ready for the color development step.

The color development steps are identical for both DRB and DRB1. To each well is added 10 ml of citrate buffer (100 mM Sodium Citrate, pH = 5.0) and the tray is

place on an orbital shaker for 5 minutes at approximately 50 rpm. Preferably, the color development solution is prepared during this time (no more than 10 minutes before use). The color development solution for each well is made from 10 ml of citrate buffer, 10 µl of 3% hydrogen peroxide, and 0.5 ml [TMB] chromogen solution
5 (commercially available from PECI) mixed gently (do not vortex).

The tray is removed from the orbital shaker, the citrate buffer is aspirated, and 10 ml of the freshly-made color development solution is added to each well. The tray is shielded from light beginning with the color development step using an aluminum foil covering. The strips are developed at room temperature for up to 30 minutes on an
10 orbital shaker at about 50 rpm. The development can be terminated as soon as the desired signal intensity has been reached.

After the desired signal intensity has been reached, the tray is removed from the shaker and the contents of each well are aspirated. The color development is stopped by washing with 10 ml of deionized water in each well. The water is added, the tray is
15 then shaken at room temperature for 5 minutes on an orbital shaker at about 50 rpm, and the contents of each well are slowly poured off. At least three washes should be performed.

The strips can be stored protected from light in the typing trays at between 2°C and 8°C for 2 to 3 days. The strips should be photographed wet for a permanent
20 record. Strips can also be dried and stored in the dark, though some fading can occur.

Results are interpreted by reading the pattern of blue dots on the DNA Probe strips; each blue dot indicates that a biotinylated amplified product hybridized with the immobilized probe. The internal control probe (DRB42), which detects all of the DRB alleles, is designed to produce a dot of intensity equal to or less than the intensity of
25 other positive dots on the strip. This provides a guide as to the minimum dot intensity that should be scored as a positive.

The probes of the above kit distinguish 31 HLA DRB1 types. Some of these probe-defined types are sets of related alleles. Figure 10 shows the probe hybridization pattern for each allele of the 1990 allele set. Figures 11, 12, and 13 provide the
30 possible interpretations for each probe hybridization pattern. Strip results can easily be interpreted directly using Figures 11-13.

A convenient alternative to manual interpretation of results is provided by a computer program which accepts the probe hybridization pattern as input, matches the input pattern to known allele patterns, and provides the possible interpretations.
35 Suitable algorithms for matching the probe hybridization pattern to the possible allele combinations include simple table lookup and decision tree algorithms. Program input

can be entered either manually or from an automated strip reader that detects the intensity of the blue hybridization dots.

As noted above, this reverse dot blot typing system does not fully discriminate among all possible types. For example, the system does not subtype DR2 serotypes.

- 5 Certain heterozygous combinations also cannot be fully resolved. All of these can be resolved with additional primer pairs for allele-specific amplifications and probes, as discussed in Example 12, below.

Because of the hybridization positions of primers CRX28 and CRX29, it is not possible to detect amino acid variations which occur at position 86 when amplifying 10 with this primer pair. Consequently, alleles which differ only at position 86 cannot be distinguished. There are seven pairs of alleles known which differ only in the present of a glycine or a valine at position 86; these allele pairs are listed below.

Allele pairs differing only at amino acid position 86

- 15 DRB1*1501 and DRB1*1502
 DRB1*0302 and DRB1*0303
 DRB1*0403 and DRB1*0407
 DRB1*0404 and DRB1*0408
 DRB1*1101 and DRB1*1104
 DRB1*1301 and DRB1*1302
20 DRB1*0802 and DRB1*0804

Example 10

General DRB1 Typing Strategy II

Example 4 describes one strategy for typing DRB1 alleles; an alternate strategy is described here. In order to obtain complete discrimination of all 45+ alleles at the 25 DRB1 locus, a two stage assay is used. The first stage involves amplification of all samples with the DRB general primers, GH46 and GH50. The resulting PCR product is immobilized and probed (as in Example 4) with a first panel of probes to determine which allele specific amplifications, if any, need to be performed and the amplification products screened in the second stage. The amplification and hybridization protocols 30 are essentially as described in Example 4. Interpretation of the hybridization pattern by reference to the allele specificities listed with each probe panel.

First Stage Typing

The first panel of probes is a similar to the probe panel shown in Table 4, differing by only a few probes. In the allele specificities shown in the probe panels 35 below, an "X" as the last digit of an allele designation indicates that all alleles beginning

with the specified number are recognized. For example, 030X is equivalent to 0301, 0302, and 0303.

First Panel of HLA DRB Typing SSO Probes

| | <u>Probe</u> | <u>SEQ ID NO:</u> | <u>Epitope</u> | <u>Alleles</u> | <u>Wash (SSPE, °C.)</u> |
|----|--------------|-------------------|----------------|--|-------------------------|
| 5 | CRX33 | SEQ ID NO: 69 | "W-L-F" | 010X | 0.4X, 42 |
| | GH104 | SEQ ID NO: 90 | "W-P-R" | 150X, 160X | 0.2X, 42 |
| | GH56 | SEQ ID NO: 86 | "YSTS" | 030X, 110X, 130X, 1401, 1402 | 0.2X, 42 |
| 10 | GH59 | SEQ ID NO: 87 | "V-H" | 040X | 0.2X, 42, 20 |
| | CRX49 | SEQ ID NO: 74 | "G-YK" | 070X | 1.0X, 42 |
| 15 | GH102 | SEQ ID NO: 89 | "YSTG" | 080X, 120X, 1404 | 0.1X, 42 |
| | GH111 | SEQ ID NO: 92 | "K-D-F" | 0901 | 0.4X, 42 |
| | CRX34 | SEQ ID NO: 70 | "EV" | 1001 | 0.4X, 42 |
| | GH122 | SEQ ID NO: 93 | "E" | 110X | 0.2X, 42 |
| | CRX61 | SEQ ID NO: 80 | "S" | 0405, 0409, 0410, 0411, 0801, 0803, 1304, 1305 | 0.1X, 42 |
| 20 | CRX23 | SEQ ID NO: 66 | "A-H" | 1401, 1404 | 0.1X, 42 |
| | CRX06 | SEQ ID NO: 61 | "I-DE" | 0103, 0402, 1102, 1301, 1302, 1304 | 0.1X, 42 |
| | CRX35 | SEQ ID NO: 71 | "F-DR" | 1601, 1101, 1104, 1305, 0801, 0802, 0804, 1202 | 0.2X, 42 |
| | CRX68 | SEQ ID NO: 84 | "F-DE" | 1103 | 0.2X, 42 |
| 25 | CRX62 | SEQ ID NO: 81 | "I-DK" | 1303 | 0.2X, 42 |
| | CRX12 | SEQ ID NO: 63 | DRB "ALL" | All | 0.2X, 42 |

The probes are hybridized and then washed for 15 minutes at 42°C, except for GH59, which is washed for 20 minutes at 42°C. All SSPE wash solutions contain 0.1% SDS. Each probe is conjugated to HRP at the 5'-end. Based on the hybridization pattern obtained from the first probe panel, a subtyping step may be necessary using allele-specific amplification.

Each of the types, DR7, DR9, and DR10 have only one identifiable allele and are identified directly from the first stage probe panel. There are actually two alleles specifying the DR7 type, DRB1*0701 and DRB1*0702. However, these two alleles differ only in the third exon and cannot be distinguished by the present methods which amplify and detect sequences from the second exon. Therefore, only one distinct allele is identifiable by the methods of the present invention.

Second Stage SubtypingDR2

Samples that contained a positive signal for W-P-R (GH104), which all type as DR2 serologically, are amplified with a primer pair specific for alleles with the W-P-R epitope, AB83 and AB60. The resulting product is probed with the following panel.

Panel of HLA DR2 Typing SSO Probes

| <u>Probe</u> | <u>SEQ ID NO:</u> | <u>Epitope</u> | <u>Alleles</u> | <u>Wash (SSPE, °C.)</u> |
|--------------|-------------------|----------------|----------------|-------------------------|
| | DRB63 | SEQ ID NO: 140 | "I-A" | 150X |
| | DRB113 | SEQ ID NO: 189 | "DR" | 1602 |
| 10 | CRX35 | SEQ ID NO: 71 | "F-DR" | 1601 |
| | CRX57 | SEQ ID NO: 78 | "V" | 1501, 1502 |
| | CRX56 | SEQ ID NO: 77 | "G" | 1502, 160X |
| | DRB196 | SEQ ID NO: 272 | "H" (30) | 1503 |
| | DRB197 | SEQ ID NO: 273 | "Y" (30) | 1501, 1502, 160X |
| 15 | GH104 | SEQ ID NO: 90 | "W-P-R" | DR2 |
| | CRX12 | SEQ ID NO: 63 | DRB "ALL" | All |
| | | | | 0.2X, 42 |

The probes shown are hybridized and then washed for 15 minutes at 42°C. All SSPE wash solutions contain 0.1% SDS. Each probe is conjugated to HRP at the 5'-end.

DR3, DR5, DRw6

20 Samples that contained a positive signal for the YSTS epitope with probe GH56 are either DR3, DR5, or DRw6. These alleles are amplified specifically with AB82 and AB60. The alleles can then be distinguished using the probe panel shown below.

Panel of HLA DR3, 5, w6 Typing SSO Probes

| <u>Probe</u> | <u>SEQ ID NO:</u> | <u>Epitope</u> | <u>Alleles</u> | <u>Wash (SSPE, °C.)</u> |
|--------------|-------------------|----------------|----------------|-------------------------|
| 25 | CRX50 | SEQ ID NO: 75 | KGR | 030X |
| | GH125 | SEQ ID NO: 94 | Y | 0301 |
| | DRB180 | SEQ ID NO: 256 | A | 030X, 1301, 1302, |
| | | | | 1305, 1402, 1403, |
| | | | | 1405 |
| 30 | GH122 | SEQ ID NO: 93 | E | 110X |
| | CRX61 | SEQ ID NO: 80 | S | 1303, 1304 |
| | CRX23 | SEQ ID NO: 66 | A-H | 1401 |
| | CRX06 | SEQ ID NO: 61 | I-DE | 1102, 1301-02 |
| | CRX62 | SEQ ID NO: 81 | I-DK | 1303 |
| 35 | CRX68 | SEQ ID NO: 84 | FDE | 1103 |
| | CRX35 | SEQ ID NO: 71 | FDR | 1101, 1104, 1305 |
| | | | | 0.2X, 42 |

| <u>Probe</u> | <u>SEQ ID NO:</u> | <u>Epitope</u> | <u>Alleles</u> | <u>Wash (SSPE, °C.)</u> |
|--------------|-------------------|----------------|--|-------------------------|
| CRX04 | SEQ ID NO: 60 | R | 1402 | 0.1X, 42 |
| CRX57 | SEQ ID NO: 78 | V | 0301, 0303, 1102, 1104, 1301, 1304, | 0.2X, 42 |
| 5 | | | 1305, 1405 | |
| CRX56 | SEQ ID NO: 77 | G | 0302, 1101, 1302, 1303, 1305, 1402, 1403 | 0.2X, 42 |
| | GH56 | SEQ ID NO: 86 | YSTS | DR3, DR5, DRw6 0.2X, 42 |
| 10 | CRX12 | SEQ ID NO: 63 | DRB "ALL" | ALL 0.2X, 42 |

The probes shown are hybridized and then washed for 15 minutes at 42°C. All SSPE wash solutions contain 0.1% SDS. Each probe is conjugated to HRP at the 5'-end.

DR4

15 Samples that contained a positive signal for the VH epitope with probe GH59 are DR4. These alleles are amplified specifically with AB54 and AB60. The alleles can then be distinguished using the probe panel shown below.

| <u>Probe</u> | <u>Epitope</u> | <u>Allele</u> | <u>Wash</u> |
|--------------|----------------|---|-------------|
| CRX61 | S | 0405, 0409, 0410, 0411 | 0.2X, 42 |
| 20 CRX64 | K | 0401, 0409 | 2X, 40 |
| CRX04 | R | 0403, 0404, 0405, 0406, 0407, 0408, 0410, 0411 | 0.1X, 42 |
| CRX15 | R-E | 0403, 0406, 0407, 0411 | 0.4X, 55 |
| CRX06 | IDE | 0402 | |
| 25 CRX57 | V | 0402, 0403, 0404, 0406, 0410, 0411 | 0.2X, 42 |
| CRX56 | G | 0401, 0405, 0407, 0408, 0409 | 0.2X, 42 |
| GH59 | V-H | DR4 | 0.2X, 42 |
| 30 CRX12 | DRB"ALL" | ALL | 0.2X, 42 |

The probes shown are hybridized and then washed for 15 minutes at 42°C with the exception of GH59, which is washed for 20 minutes. All SSPE wash solutions contain 0.1% SDS. Each probe is conjugated to HRP at the 5'-end.

DR1, DRw8, and DRw12

Samples that contained a positive signal for the WLF epitope with probe CRX33 or positive for the YSTG epitope with probe GH102 are amplified with the DRB1-specific primers, GH46 and CRX37. This also amplifies all DRB1 alleles except DR2, DR7, and DR9. The alleles can then be distinguished using the probe panel shown below.

| <u>Probe</u> | <u>Epitope</u> | <u>Allele</u> | <u>Wash (SSPE, °C)</u> |
|--------------|----------------|---|------------------------|
| CRX33 | WLF | 010X | 0.4X, 42 |
| CRX04 | R | 0101, 0102, 0403, 0404, 10 0405, 0406, 0407, 0408, 0410, 0411 | 0.1X, 42 |
| CRX06 | IDE | 0103, 1102, 1301, 1302 | 0.2X, 42 |
| CRX57 | V | 0301, 0402, 0403, 0404, 15 0406, 0410, 0411, 1102, 1103, 1104, 1301, 1304, 1401, 1404, 1405, 0804 | 0.2X, 42 |
| DRB181 | AV | 0102, 1201, 1202 | 0.1X, 42 |
| CRX56 | G | 0101, 0103, 0302, 0401, 20 0405, 0407, 0408, 0409, 1101, 1302, 1303, 0801, 0802, 0803, 1305, 1402, 1403 | 0.2X, 42 |
| GH102 | YSTG | 0801, 0802, 0803, 0804, 25 1201, 1202; 1404 | 0.1X, 42 |
| GH54 | V-S | 1201, 1202 | 0.4X, 42 |
| CRX63 | I-DR | 1201, 0803 | 0.2X, 42 |
| CRX35 | F-DR | 0801, 0802, 1202, 1101, 30 1104, 1305 | 0.2X, 42 |
| CRX12 | DRB "ALL" | ALL | 0.2X, 42 |

30 The probes shown are hybridized and then washed for 15 minutes at 42°C. All SSPE wash solutions contain 0.1% SDS. Each probe is conjugated to HRP at the 5'-end.

Since some epitopes recognized by the probes are shared in many alleles (such as FDR), the results obtained from the hybridization pattern with the above probe panel are compared to other allele specific amplifications to determine if the probe is associated with a DR1, DRw8, or DRw12, or is associated with another allele also amplified with the DRB1-specific primers. The design of primers specific for the WLF and YSTG epitopes will simplify the interpretation of the typing results.

Example 11Allele Subtyping in Heterozygotes

- The pattern of hybridization with the probe panels of Example 10 alone may not be sufficient to unambiguously determine which alleles are present in certain heterozygous individuals. The probe hybridization pattern indicates which allele epitopes are present in the sample, but to determine the specific alleles present, it may be necessary to know which epitopes occur on which allele. Most frequently, such ambiguity does not arise because the epitope origin can usually be inferred from the limited possibilities of allelic combinations. The few cases which do arise can be resolved using allele-specific amplification.

In some DR5/DRw6 heterozygotes, the following probe pattern can result:

Probe: GH56 + GH122 + DRB180 + CRX06 + CRX35 + CRX56 + CRX57

Epitope: YSTS E DA I-DE F-DR G V

- Three different DR5/DRw6 heterozygote allele combinations produce this probe hybridization pattern; these combinations are listed below. These heterozygote allele combinations cannot be distinguished by any other probe.

DRB1*1101 and DRB1*1301

DRB1*1104 and DRB1*1302

DRB1*1102 and DRB1*1305

- To distinguish these possibilities, additional primers were designed to take advantage of the unique dimorphism at position 86. All DRB1 alleles contain either a valine or a glycine at position 86. In each of the heterozygotes listed above, one of the alleles contains a valine at position 86 and the other contains a glycine. DRB1*1301, DRB1*1104, and DRB1*1102 each contain a valine at position 86; DRB1*1101, DRB1*1302, and DRB1*1305 each contain a glycine. Amplification using either a V or G specific primer in combination with a group specific primer (W-PR, VH, or YSTS) allows single allele amplification based on the polymorphism at position 86. In this manner, one of the two alleles present in each heterozygote can be selectively amplified and determined by direct probe hybridization. The second allele can be inferred from knowledge of the first allele based on the possible allelic combinations.

- Subtyping DR5/DRw6 heterozygotes by single allele amplification was achieved using the PCR primer pairs AB82/RAP05 and AB82/RAP06. Amplification was carried out as in Example 10 except that the PCR buffer contained a final concentration of 1.0 mM MgCl₂. The thermocycler was programmed for 35 cycles, each with the following temperature profile: ramp to 94°C; 30 seconds denaturation at 94°C; 30 seconds annealing and extension at 70°C. Probe hybridization was carried out as before. The sequences of the V-specific (RAP06) and G-specific (RAP05)

primers are shown below and in the sequence listing section. Although each was used in combination with AB82, other group specific primers would be suitable.

| | <u>Primer</u> | <u>SEQ ID NO:</u> | <u>Specificity</u> | <u>Sequence</u> |
|---|---------------|-------------------|--------------------|-----------------------------|
| | RAP05 | SEQ ID NO: 312 | G (86) | 5'-CGCTGCACTGTGAAGCTCTCACCA |
| 5 | RAP06 | SEQ ID NO: 313 | V (86) | 5'-CGCTGCACTGTGAAGCTCTCCACA |

Example 12

DRB Two-Stage Typing Kit

The typing kit described here is designed to uniquely identify the greatest number of types possible. The primers and probes are designed to provide rapid yet 10 complete typing for the DRB1 and DRB3 loci in a reverse dot blot format. In order to obtain complete discrimination of all 45+ alleles at the DRB1 locus and the 3 alleles at the DRB3 locus, a two stage assay is used. The first stage involves amplification of all samples with the DRB general primers, DRB27 (SEQ ID NO: 105) and DRB28 (SEQ ID NO: 106). The resulting PCR product is screened against a strip with 13 probes to 15 determine which allele specific amplifications, if any, need to be done and screened in the second stage.

Amplification and hybridization are carried out as in Example 9. The amplification product from the DRB general amplification is screened against a panel of probes containing eight probes specific for the first region of variability in the 20 DRB1 locus, four probes specific for the three DRB3 alleles (52a, 52b, 52c), and one control probe. Additional probes can be added to this strip to type for the DRB5 locus which has three alleles and is found on DR2 haplotypes. The specific hybridization regions and types recognized are listed below.

| | <u>Type recognized</u> | <u>Sequence Probed</u> |
|----|------------------------|------------------------|
| 1 | DR1 | WLF |
| 2 | DR2 | WPR |
| 3 | DR3, 5, 6 | YSTS |
| 5 | DR4 | VH |
| 5 | DR7 | GYK |
| 6 | DR8, 12 | YSTG |
| 7 | DR9 | KDF |
| 8 | DR10 | EV |
| 10 | 52a | LRS |
| 10 | 52b, 52c | LLS |
| 11 | 52b | to be designed |
| 12 | 52c | to be designed |
| 13 | All | TELGRP |

15 The DRB3 type is determined from the results of this assay and, based on the results of this assay, it is determined which allele-specific amplifications (ASAs), if any, need to be done. Primers for five different ASAs are provided, though the most needed for typing any one sample is two. The same 3' primer, AB60, for example, can be used for all five ASAs and typically would be included as a component of the
 20 PCR reaction mix provided in a kit. Amplification specificity is conferred by the 5' primers. The DRB type amplified and the epitope recognized by each of the 5' primers are listed below. Primers 2, 3, and 4 have been designed and tested extensively and are discussed in the examples, above.

| | <u>Type Amplified</u> | <u>Hybridization Sequence</u> |
|----|-----------------------|-------------------------------|
| 25 | 1 DR1 | WLF |
| | 2 DR2 | WPR |
| | 3 DR3, 5, 6 | YSTS |
| | 4 DR4 | VH |
| | 5 DR8, 12 | YSTG |

30 The second stage involves a series of probes which permit fine typing of the amplified products from the ASAs. In one embodiment, all probes are attached to a single strip and this single strip is used to type the product from all ASAs. Probes specific to the following regions are sufficient for typing the results of the ASAs.

| <u>Region</u> | <u>Epitopes</u> |
|------------------|--|
| 57-60 | DAE, S, E, A-H, V-S |
| 67-74 | R, I-DE, I-A, F-DR, DR, KGR, K, R-E/RR-E, F-DE, I-DK, I-DR, DR-L |
| 5 85-86 G, V, AV | |
| Other | E (a.a. 20), H (a.a. 30), Y (a.a. 26), S (a.a. 37) |
| | The "other" region polymorphisms are very rare in Caucasian populations. |
| | However, an important goal of the present method is to be able to type non-Caucasian populations. Additional probes at these positions can be included to deal with possible |
| 10 | ambiguities in recognizing heterozygotes versus homozygotes. |

Table 9

Alleles not in the 1989 Allele Set

DRB1*0303 (SEQ ID NO: 6): ** *** *** *** *** TAC TCT ACG TCT GAG
 TGT CAT TTC TTC AAT GGG ACG GAG CGG GTG CGG TTC CTG GAG AGA TAC TTC
 15 CAT AAC CAG GAG GAG AAC GTG CGC TTC GAC AGC GAC GTG GGG GAG TAC CGG
 GCG GTG ACG GAG CTG GGG CGG CCT GAT GCC GAG TAC TGG AAC AGC CAG AAG
 GAC CTC CTG GAG CAG AAG CGG GGC CGG GTG GAC AAC TAC TGC AGA CAC AAC
 TAC GGG GTT GTG GAG AGC TTC ACA GTG CAG CGG CGA

20 DRB1*0409 (SEQ ID NO: 15): CA CGT TTC TTG GAG CAG GTT AAA CAT GAG
 TGT CAT TTC TTC AAC GGG ACG GAG CGG GTG CGG TTC CTG GAC AGA TAC TTC
 TAT CAC CAA GAG GAG TCA GTG CGC TTC GAC AGC GAC GTG GGG GAG TAC CGG
 GCG GTG ACG GAG CTG GGG CGG CCT AGC GCC GAG TAC TGG AAC AGC CAG AAG
 GAC CTC CTG GAG CAG AAG CGG GGC CGG GTG GAC ACC TAC TGC AGA CAC AAC
 25 TAC GGG GTT GGT GAG AGC TTC ACA GTG CAG CGG CGA

DRB1*0410 (SEQ ID NO: 16): CA CGT TTC TTG GAG CAG GTT AAA CAT GAG
 TGT CAT TTC TTC AAC GGG ACG GAG CGG GTG CGG TTC CTG GAC AGA TAC TTC
 TAT CAC CAA GAG GAG TCA GTG CGC TTC GAC AGC GAC GTG GGG GAG TAC CGG
 30 GCG GTG ACG GAG CTG GGG CGG CCT AGC GCC GAG TAC TGG AAC AGC CAG AAG
 GAC CTC CTG GAG CAG AGG CGG GGC CGG GTG GAC ACC TAC TGC AGA CAC AAC
 TAC GGG GTT GTG GAG AGC TTC ACA GTG CAG CGG CGA

DRB1*0411 (SEQ ID NO: 17): CA CGT TTC TTG GAG CAG GTT AAA CAT GAG
TGT CAT TTC TTC AAC GGG ACG GAG CGG GTG CGG TTC CTG GAC AGA TAC TTC
TAT CAC CAA GAG GAG TCA GTG CGC TTC GAC AGC GAC GTG GGG GAG TAC CGG
CGG GTG ACG GAG CTG GGG CGG CCT AGC GCC GAG TAC TGG AAC AGC CAG AAG
5 GAC CTC CTG GAG CAG AGG CGG GCC GAG GTG GAC ACC TAC TGC AGA CAC AAC
TAC GGG GTT GTG GAG AGC TTC ACA GTG CAG CGG CGA

DRB1*0804 (SEQ ID NO: 22): CA CGT TTC TTG GAG TAC TCT ACG GGT GAG
TGT TAT TTC TTC AAT GGG ACG GAG CGG GTG CGG TTC CTG GAC AGA TAC TTC
10 TAT AAC CAA GAG GAG TAC GTG CGC TTC GAC AGC GAC GTG GGG GAG TAC CGG
GCG GTG ACG GAG CTG GGG CGG CCT GAT GCC GAG TAC TGG AAC AGC CAG AAG
GAC TTC CTG GAA GAC AGG CGG GCC CTG GTG GAC ACC TAC TGC AGA CAC AAC
TAC GGG GTT GTG GAG AGC TTC ACA GTG CAG CGG CGA

15 DRB1*1105 (SEQ ID NO: 29): CA CGT TTC TTG GAG TAC TCT ACG GGT GAG
TGT CAT TTC TTC AAT GGG ACG GAG CGG GTG CGG TTC CTG GAC AGA TAC TTC
TAT AAC CAA GAG GAG TAC GTG CGC TTC GAC AGC GAC GTG GGG GAG TTC CGG
GCG GTG ACG GAG CTG GGG CGG CCT GAT GAG GAG TAC TGG AAC AGC CAG AAG
GAC TTC CTG GAA GAC AGG CGG GCC GCG GTG GAC ACC TAC TGC AGA CAC AAC
20 TAC GGG GTT GGT GAG AGC TTC ACA GTG CAG CGG CGA

25 DRB1*1202 (SEQ ID NO: 31): CA CGT TTC TTG GAG TAC TCT ACG GGT GAG
TGT TAT TTC TTC AAT GGG ACG GAG CGG GTG CGG TTA CTG GAG AGA CAC TTC
CAT AAC CAG GAG GAG CTC CTG CGC TTC GAC AGC GAC GTG GGG GAG TTC CGG
GCG GTG ACG GAG CTG GGG CGG CCT GTC GCC GAG TCC TGG AAC AGC CAG AAG
GAC TTC CTG GAA GAC AGG CGC GCC GCG GTG GAC ACC TAT TGC AGA CAC AAC
TAC GGG GCT GTG GAG AGC TTC ACA GTG CAG CGG CGA

30 DRB1*1304 (SEQ ID NO: 35): CA CGT TTC TTG GGG TAC TCT ACG TCT GAG
TGT CAT TTC TTC AAT GGG ACG GAG CGG GTG CGG TTC CTG GAC AGA TAC TTC
TAT AAC CAA GAG GAG TAC GTG CGC TTC GAC AGC GAC GTG GGG GAG TTC CGG
GCG GTG ACG GAG CTG GGG CGG CCT AGC GCC GAG TAC TGG AAC AGC CAG AAG
GAC ATC CTG GAA GAC GAG CGG GCC GCG GTG GAC ACC TAC TGC AGA CAC AAC
TAC GGG GTT GTG GAG AGC TTC ACA GTG CAG CGG CGA

DRB1*1403 (SEQ ID NO: 39): CA CGT TTC TTG GGG TAC TCT ACG TCT GAG
TGT CAT TTC TTC AAT GGG ACG GAG CGG GTG CGG TTC CTG GAG AGA TAC TTC
CAT AAC CAG GAG GAG AAC GTG CGC TTC GAC AGC GAC GTG GGG GAG TAC CGG
5 GCG GTG ACG GAG CTG GGG CGG CCT GAT GCC GAG TAC TGG AAC AGC CAG AAG
GAC CTC CTG GAA GAC AGG CGG GCC CTG GTG GAC ACC TAC TGC AGA CAC AAC
TAC GGG GTT GGT GAG AGC TTC ACA GTG CAG CGG CGA

DRB1*1405 (SEQ ID NO: 41): CA CGT TTC TTG GGG TAC TCT ACG TCT GAG
TGT CAA TTC TTC AAT GGG ACG GAG CGG GTG CGG TTC CTG GAC AGA TAC TTC
10 CAT AAC CAG GAG GAG TTC GTG CGC TTC GAC AGC GAC GTG GGG GAG TAC CGG
GCG GTG ACG GAG CTG GGG CGG CCT GAT GCT GAG TAC TGG AAC AGC CAG AAG
GAC CTC CTG GAG CGG AGG CGG GCC GAG GTG GAC ACC TAT TGC AGA CAC AAC
TAC GGG GTT GTG GAG AGC TTC ACA GTG CAG CGG CGA

15 DRB1*1503 (SEQ ID NO: 44): CA CGT TTC CTG TGG CAG CCT AAG AGG GAG
TGT CAT TTC TTC AAT GGG ACG GAG CGG GTG CGG TTC CTG GAC AGA CAC TTC
TAT AAC CAG GAG GAG TCC GTG CGC TTC GAC AGC GAC GTG GGG GAG TTC CGG
GCG GTG ACG GAG CTG GGG CGG CCT GAC GCT GAG TAC TGG AAC AGC CAG AAG
GAC ATC CTG GAG CAG CGG CGG GCC GCG GTG GAC ACC TAC TGC AGA CAC AAC
20 TAC GGG GTT GTG GAG AGC TTC ACA GTG CAG CGG CGA

We Claim

1. A method for determining the DRbeta DNA type of nucleic acid in a sample, which method comprises:

- (a) amplifying any nucleic acids in the sample that contain a DRbeta gene second exon;
- 5 (b) hybridizing said nucleic acid amplified in step (a) to a first panel of oligonucleotide probes under conditions such that said probes hybridize only to exactly complementary sequences greater than ten nucleotides in length;
- (c) amplifying a specific subset of nucleic acids in the sample that contain 10 DRbeta gene second exon sequences;
- (d) hybridizing said nucleic acid amplified in step (c) with a second panel of oligonucleotide probes under conditions such that said probes hybridize only to exactly complementary sequences greater than ten nucleotides in length; and
- 15 (e) determining from the pattern of probe hybridization in steps (b) and (d) the DRbeta alleles from which the DRbeta DNA in said sample originates.

2. The method of Claim 1, wherein the amplifying of steps (a) and (c) is carried out by a polymerase chain reaction with a pair of oligonucleotide primers, and wherein the primer pair used in step (a) is different from the primer pair used in step 20 (c).

3. The method of Claim 2, wherein the primers used in step (a) are GH46 and GH50, and the primers used in step (c) are either GH46 and CRX37 or GH46 and DRB30.

25 4. The method of Claim 1, wherein said first and second panel of oligonucleotide probes each comprise two or more probes, and wherein said probes can hybridize to DRbeta gene second exon sequences, which sequences are selected from the group consisting of sequences that encode amino acid residues 9 to 16, amino acid residues 25 to 34, amino acid residues 67 to 74, and amino acid residue 86.

30

5. The method of Claim 4, wherein the first panel of oligonucleotide probes comprises two or more probes selected from the group consisting of probes CRX60 (SEQ ID NO: 79), GH105 (SEQ ID NO: 91), GH104 (SEQ ID NO: 90), GH59 (SEQ ID NO: 57), CRX06 (SEQ ID NO: 61), GH122 (SEQ ID NO: 93), CRX23 (SEQ ID NO: 66), CRX35 (SEQ ID NO: 71), CRX49 (SEQ ID NO: 74), GH102 (SEQ ID NO: 89), GH111 (SEQ ID NO: 92), CRX34 (SEQ ID NO: 70), CRX04 (SEQ ID NO: 60), GH56 (SEQ ID NO: 86), CRX68 (SEQ ID NO: 84), and CRX12 (SEQ ID NO: 63).

10 6. The method of Claim 4, wherein the second panel of oligonucleotide probes comprises two or more probes selected from the group consisting of probes GH125 (SEQ ID NO: 94), CRX50 (SEQ ID NO: 68), CRX53 (SEQ ID NO: 76), CRX15 (SEQ ID NO: 64), CRX62 (SEQ ID NO: 81), CRX63 (SEQ ID NO: 82), CRX61 (SEQ ID NO: 80), GH54 (SEQ ID NO: 85), CRX56 (SEQ ID NO: 77), CRX57 (SEQ ID NO: 78), and CRX12 (SEQ ID NO: 63).

15 7. The method of Claim 2, wherein the primers used in step (a) are GH46 (SEQ ID NO: 67) and GH50 (SEQ ID NO: 68), the primers used in step (c) are GH46 and CRX37 (SEQ ID NO: 73), the first panel of oligonucleotide probes comprises probes CRX60 (SEQ ID NO: 79), GH105 (SEQ ID NO: 91), GH104 (SEQ ID NO: 90), GH59 (SEQ ID NO: 57), CRX06 (SEQ ID NO: 61), GH122 (SEQ ID NO: 93), CRX23 (SEQ ID NO: 66), CRX35 (SEQ ID NO: 71), CRX49 (SEQ ID NO: 74), GH102 (SEQ ID NO: 89), GH111 (SEQ ID NO: 92), CRX34 (SEQ ID NO: 70), CRX04 (SEQ ID NO: 60), GH56 (SEQ ID NO: 86), CRX68 (SEQ ID NO: 84), and CRX12 (SEQ ID NO: 63), and the second panel of oligonucleotide probes comprises probes GH125 (SEQ ID NO: 94), CRX50 (SEQ ID NO: 68), CRX53 (SEQ ID NO: 76), CRX15 (SEQ ID NO: 64), CRX62 (SEQ ID NO: 81), CRX63 (SEQ ID NO: 82), CRX61 (SEQ ID NO: 80), GH54 (SEQ ID NO: 85), CRX56 (SEQ ID NO: 77), CRX57 (SEQ ID NO: 78), and CRX12 (SEQ ID NO: 63).

30 8. The method of Claim 1, wherein said first and second panels of oligonucleotide probes are fixed to a solid support, each probe located in a discrete and distinct location on said solid support.

35 9. A kit for performing the method of Claim 2, which kit comprises the primers used in steps (a) and (c) and the first and second panel of oligonucleotide probes.

10. The kit of Claim 9, which kit comprises primers GH46 (SEQ ID NO: 67),
GH50 (SEQ ID NO: 68), and CRX37 (SEQ ID NO: 73), two or more oligonucleotide
probes selected from the group consisting of probes CRX60 (SEQ ID NO: 79), GH105
(SEQ ID NO: 91), GH104 (SEQ ID NO: 90), GH59 (SEQ ID NO: 57), CRX06 (SEQ
5 ID NO: 61), GH122 (SEQ ID NO: 93), CRX23 (SEQ ID NO: 66), CRX35 (SEQ ID
NO: 71), CRX49 (SEQ ID NO: 74), GH102 (SEQ ID NO: 89), GH111 (SEQ ID NO:
92), CRX34 (SEQ ID NO: 70), CRX04 (SEQ ID NO: 60), GH56 (SEQ ID NO: 86),
CRX68 (SEQ ID NO: 84), and CRX12 (SEQ ID NO: 63), and two or more probes
selected from the group consisting of probes GH125 (SEQ ID NO: 94), CRX50 (SEQ
10 ID NO: 68), CRX53 (SEQ ID NO: 76), CRX15 (SEQ ID NO: 64), CRX62 (SEQ ID
NO: 81), CRX63 (SEQ ID NO: 82), CRX61 (SEQ ID NO: 80), GH54 (SEQ ID NO:
85), CRX56 (SEQ ID NO: 77), CRX57 (SEQ ID NO: 78), and CRX12 (SEQ ID NO:
63).
- 15 11. The kit of Claim 10, which kit comprises primers GH46 (SEQ ID NO:
67), GH50 (SEQ ID NO: 68), and CRX37 (SEQ ID NO: 73), a first panel of
oligonucleotide probes comprising probes CRX60 (SEQ ID NO: 79), GH105 (SEQ ID
NO: 91), GH104 (SEQ ID NO: 90), GH59 (SEQ ID NO: 57), CRX06 (SEQ ID NO:
61), GH122 (SEQ ID NO: 93), CRX23 (SEQ ID NO: 66), CRX35 (SEQ ID NO: 71),
20 CRX49 (SEQ ID NO: 74), GH102 (SEQ ID NO: 89), GH111 (SEQ ID NO: 92),
CRX34 (SEQ ID NO: 70), CRX04 (SEQ ID NO: 60), GH56 (SEQ ID NO: 86),
CRX68 (SEQ ID NO: 84), and CRX12 (SEQ ID NO: 63), and a second panel of
oligonucleotide probes comprising probes GH125 (SEQ ID NO: 94), CRX50 (SEQ ID
NO: 68), CRX53 (SEQ ID NO: 76), CRX15 (SEQ ID NO: 64), CRX62 (SEQ ID NO:
25 81), CRX63 (SEQ ID NO: 82), CRX61 (SEQ ID NO: 80), GH54 (SEQ ID NO: 85),
CRX56 (SEQ ID NO: 77), CRX57 (SEQ ID NO: 78), and CRX12 (SEQ ID NO: 63).

12. A method for determining whether a sample comprises nucleic acid from the DRB1 allele of a serological type selected from the group consisting of the DR1, DR2, DR4, DR7, DRw9, and DRw10 types, which method comprises:

- (a) amplifying any DRB1 nucleic acid in the sample; and
5 (b) hybridizing said nucleic acid amplified in step (a) to a panel of oligonucleotide probes under conditions such that said probes hybridize only to exactly complementary sequences greater than ten nucleotides in length, wherein said probes hybridize to unique polymorphic sequences encoding amino acid residues 9 to 16 of the DRB1 gene second exon.

10

13. The method of Claim 12, wherein said amplifying step is carried out in a polymerase chain reaction with primers GH46 and GH50 and said panel of probes comprises two or more probes selected from the group consisting of probes CRX60 (SEQ ID NO: 79), GH105 (SEQ ID NO: 91), GH104 (SEQ ID NO: 90), GH59 (SEQ 15 ID NO: 57), CRX06 (SEQ ID NO: 61), GH122 (SEQ ID NO: 93), CRX23 (SEQ ID NO: 66), CRX35 (SEQ ID NO: 71), CRX49 (SEQ ID NO: 74), GH102 (SEQ ID NO: 89), GH111 (SEQ ID NO: 92), CRX34 (SEQ ID NO: 70), CRX04 (SEQ ID NO: 60), GH56 (SEQ ID NO: 86), CRX68 (SEQ ID NO: 84), and CRX12 (SEQ ID NO: 63).

20

14. A method for determining whether a sample contains nucleic acid from a DRB1 allele selected from the group consisting of alleles 0101, 0102, 0103, 0301, 0302, 0401, 0402, 0403, 0404, 0405, 0406, 0407, 0408, 0701, 0801, 0802, 0803, 0901, 1001, 1101, 1102, 1103, 1104, 1201, 1301, 1302, 1303, 1401, 1402, DR2, DR "PEV", and for determining the allele from which the nucleic acid originates, which 25 method comprises:

- (a) amplifying any DRB1 nucleic acid in said sample; and
5 (b) hybridizing said nucleic acid amplified in step a with a panel of oligonucleotide probes under conditions such that said probes hybridize only to exactly complementary sequences greater than ten nucleotides in length.

15. The method of Claim 14, wherein said panel of oligonucleotide probes comprises two or more probes selected from the group consisting of probes CRX04 (SEQ ID NO: 60), CRX06 (SEQ ID NO: 61), CRX15 (SEQ ID NO: 64), CRX23 (SEQ ID NO: 66), CRX34 (SEQ ID NO: 70), CRX35 (SEQ ID NO: 71), CRX49 (SEQ ID NO: 74), CRX50 (SEQ ID NO: 50), CRX53 (SEQ ID NO: 76), CRX56 (SEQ ID NO: 77), CRX57 (SEQ ID NO: 78), CRX60 (SEQ ID NO: 79), CRX61 (SEQ ID NO: 80), CRX62 (SEQ ID NO: 81), CRX63 (SEQ ID NO: 82), CRX68 (SEQ ID NO: 84), GH54 (SEQ ID NO: 85), GH56 (SEQ ID NO: 86), GH59 (SEQ ID NO: 87), GH102 (SEQ ID NO: 89), GH105 (SEQ ID NO: 91), GH111 (SEQ ID NO: 92), GH122 (SEQ ID NO: 93), and GH125 (SEQ ID NO: 94).

16. The method of Claim 15, wherein said panel of oligonucleotide probes comprises probes CRX04 (SEQ ID NO: 60), CRX06 (SEQ ID NO: 61), CRX15 (SEQ ID NO: 64), CRX23 (SEQ ID NO: 66), CRX34 (SEQ ID NO: 70), CRX35 (SEQ ID NO: 71), CRX49 (SEQ ID NO: 74), CRX50 (SEQ ID NO: 50), CRX53 (SEQ ID NO: 76), CRX56 (SEQ ID NO: 77), CRX57 (SEQ ID NO: 78), CRX60 (SEQ ID NO: 79), CRX61 (SEQ ID NO: 80), CRX62 (SEQ ID NO: 81), CRX63 (SEQ ID NO: 82), CRX68 (SEQ ID NO: 84), GH54 (SEQ ID NO: 85), GH56 (SEQ ID NO: 86), GH59 (SEQ ID NO: 87), GH102 (SEQ ID NO: 89), GH105 (SEQ ID NO: 91), GH111 (SEQ ID NO: 92), GH122 (SEQ ID NO: 93), and GH125 (SEQ ID NO: 94).

17. A method for determining the serological DR type of a sample, wherein said type is selected from the group consisting of DR1 Dw1; DR1 Dw "BON"; DR2; DR4 Dw4; DR4 Dw10; DR4 Dw13, DR4 Dw14, DR4 Dw15; DR3, DRw11, DRw6; DRw11.1; DRw11.2; DRw12, DRw8.3; DRw13; DRw14 Dw16; DRw14 Dw9; DR7; DRw8.1, DRw8.2; DR9; and DRw10, which method comprises:

- (a) amplifying any DRB1 nucleic acid in said sample; and
- (b) hybridizing said nucleic acid amplified in step a with a panel of oligonucleotide probes under conditions such that said probes hybridize only to exactly complementary sequences greater than ten nucleotides in length.

18. The method of Claim 17, wherein said panel of oligonucleotide probes comprises two or more probes selected from the group consisting of probes CRX04 (SEQ ID NO: 60), CRX06 (SEQ ID NO: 61), CRX23 (SEQ ID NO: 66), CRX34 (SEQ ID NO: 70), CRX35 (SEQ ID NO: 71), CRX49 (SEQ ID NO: 74), CRX53 (SEQ ID NO: 76), CRX60 (SEQ ID NO: 79), CRX63 (SEQ ID NO: 82), GH56 (SEQ ID NO: 86), GH59 (SEQ ID NO: 87), GH102 (SEQ ID NO: 89), GH105 (SEQ ID NO: 91), GH111 (SEQ ID NO: 92), and GH122 (SEQ ID NO: 93).

19. The method of Claim 18, wherein said panel of oligonucleotide probes comprises the group consisting of probes CRX04 (SEQ ID NO: 60), CRX06 (SEQ ID NO: 61), CRX23 (SEQ ID NO: 66), CRX34 (SEQ ID NO: 70), CRX35 (SEQ ID NO: 71), CRX49 (SEQ ID NO: 74), CRX53 (SEQ ID NO: 76), CRX60 (SEQ ID NO: 79), CRX63 (SEQ ID NO: 82), GH56 (SEQ ID NO: 86), GH59 (SEQ ID NO: 87), GH102 (SEQ ID NO: 89), GH105 (SEQ ID NO: 91), GH111 (SEQ ID NO: 92), and GH122 (SEQ ID NO: 93).

20. An oligonucleotide primer selected from the group consisting of AB54 (SEQ ID NO: 56), AB82 (SEQ ID NO: 58), AB83 (SEQ ID NO: 59), DB259 (SEQ ID NO: 95), DB260 (SEQ ID NO: 96), DRB17 (SEQ ID NO: 73), DRB30 (SEQ ID NO: 107), DRB152 (SEQ ID NO: 228), CRX11 (SEQ ID NO: 62), and CRX37 (SEQ ID NO: 73).

21. An oligonucleotide probe selected from the group consisting of DRB19 (SEQ ID NO: 97), DRB20 (SEQ ID NO: 98), DRB21 (SEQ ID NO: 99), DRB27 (SEQ ID NO: 105), DRB31 (SEQ ID NO: 108), DRB32 (SEQ ID NO: 109), DRB33 (SEQ ID NO: 110), DRB34 (SEQ ID NO: 111), DRB35 (SEQ ID NO: 112), DRB36 (SEQ ID NO: 113), DRB37 (SEQ ID NO: 114), DRB42 (SEQ ID NO: 119), DRB45 (SEQ ID NO: 122), DRB46 (SEQ ID NO: 123), DRB48 (SEQ ID NO: 125), DRB60 (SEQ ID NO: 137), DRB62 (SEQ ID NO: 139), DRB63 (SEQ ID NO: 140), DRB72 (SEQ ID NO: 148), DRB84 (SEQ ID NO: 160), DRB90 (SEQ ID NO: 166), DRB91 (SEQ ID NO: 167), DRB95 (SEQ ID NO: 171), DRB100 (SEQ ID NO: 176), DRB101 (SEQ ID NO: 177), DRB102 (SEQ ID NO: 178), DRB103 (SEQ ID NO: 179), DRB107 (SEQ ID NO: 183), DRB108 (SEQ ID NO: 184), DRB109 (SEQ ID NO: 185), DRB112 (SEQ ID NO: 188), DRB113 (SEQ ID NO: 189), and DRB118 (SEQ ID NO: 194).

22. The method of Claim 1, wherein said first panel of probes comprises two or more probes selected from the group consisting of probes DRB01 (SEQ ID NO: 79), GH104 (SEQ ID NO: 90), DRB46 (SEQ ID NO: 123), DRB48 (SEQ ID NO: 125), DRB207 (SEQ ID NO: 283), GH102 (SEQ ID NO: 89), DRB209 (SEQ ID NO: 285),
5 DRB20 (SEQ ID NO: 98), DRB102 (SEQ ID NO: 178), DRB112 (SEQ ID NO: 188), DRB07 (SEQ ID NO: 84), and DRB42 (SEQ ID NO: 119).

23. The method of Claim 1, wherein said second panel of probes comprises two or more probes selected from the group consisting of probes DRB223 (SEQ ID NO: 299), DRB37 (SEQ ID NO: 144), DRB203 (SEQ ID NO: 279), DRB163 (SEQ ID NO: 239), DRB118 (SEQ ID NO: 194), DRB02 (SEQ ID NO: 61), DRB38 (SEQ ID NO: 115), DRB222 (SEQ ID NO: 298), DRB232 (SEQ ID NO: 308), DRB136 (SEQ ID NO: 212), DRB198 (SEQ ID NO: 274), and DRB42 (SEQ ID NO: 119).
10

15 24. The method of Claim 2, wherein the primers used in step (a) are CRX28 (SEQ ID NO: 67) and CRX29 (SEQ ID NO: 68), the primers used in step (c) are CRX28 and CRX37 (SEQ ID NO: 73), the first panel of oligonucleotide probes comprises probes DRB01 (SEQ ID NO: 79), GH104 (SEQ ID NO: 90), DRB46 (SEQ ID NO: 123), DRB48 (SEQ ID NO: 125), DRB207 (SEQ ID NO: 283), GH102 (SEQ ID NO: 89), DRB209 (SEQ ID NO: 285), DRB20 (SEQ ID NO: 98), DRB102 (SEQ ID NO: 178), DRB112 (SEQ ID NO: 188), DRB07 (SEQ ID NO: 84), and DRB42 (SEQ ID NO: 119), and the second panel of oligonucleotide probes comprises probes DRB223 (SEQ ID NO: 299), DRB37 (SEQ ID NO: 144), DRB203 (SEQ ID NO: 279), DRB163 (SEQ ID NO: 239), DRB118 (SEQ ID NO: 194), DRB02 (SEQ ID NO: 61),
20 DRB38 (SEQ ID NO: 115), DRB222 (SEQ ID NO: 298), DRB232 (SEQ ID NO: 308), DRB136 (SEQ ID NO: 212), DRB198 (SEQ ID NO: 274), and DRB42 (SEQ ID NO: 119).
25

25. A method for determining whether a sample contains nucleic acid from a DRB1 allele selected from the group consisting of alleles 0101, 0102, 0103, 1501, 1502, 1503, 1504, 1601, 1602, 0301, 0302, 0303, 0401, 0402, 0403, 0404, 0405, 0406, 0407, 0408, 0409, 04010, 04011, 1101, 1102, 1103, 1104, BUGS, 1301, 5 1302, 1303, 1304, 1305, 1401, 1402, 1403, 1404, 1405, 0701, 0801, 0802, 0803, 0804, 0901, 1001, 1201, and 1202, and for determining the allele from which the nucleic acid originates, which method comprises:
- (a) amplifying any DRB1 nucleic acid in said sample; and
 - (b) hybridizing said nucleic acid amplified in step a with a panel of 10 oligonucleotide probes under conditions such that said probes hybridize only to exactly complementary sequences greater than ten nucleotides in length.

26. The method of Claim 1, wherein the first panel of oligonucleotide probes comprises two or more probes selected from the group consisting of probes CRX33 15 (SEQ ID NO: 69), GH104 (SEQ ID NO: 90), GH56 (SEQ ID NO: 86), GH59 (SEQ ID NO: 87), CRX49 (SEQ ID NO: 74), GH102 (SEQ ID NO: 89), GH111 (SEQ ID NO: 92), CRX34 (SEQ ID NO: 70), GH122 (SEQ ID NO: 93), CRX61 (SEQ ID NO: 80), CRX23 (SEQ ID NO: 66), CRX06 (SEQ ID NO: 61), CRX35 (SEQ ID NO: 71), CRX68 (SEQ ID NO: 84), and CRX62 (SEQ ID NO: 81).

27. The method of Claim 1, wherein the second panel of probes comprises two or more probes selected from a third panel of probes, wherein said third panel is selected from the group consisting of panels GH104 (SEQ ID NO: 90), DRB63 (SEQ ID NO: 140), DRB113 (SEQ ID NO: 189), CRX35 (SEQ ID NO: 71), CRX57 (SEQ 5 ID NO: 78), CRX56 (SEQ ID NO: 77), DRB196 (SEQ ID NO: 272), DRB197 (SEQ ID NO: 273), DRB215 (SEQ ID NO: 291), and DRB216 (SEQ ID NO: 292); CRX50 (SEQ ID NO: 75), GH125 (SEQ ID NO: 94), DRB180 (SEQ ID NO: 256), GH122 (SEQ ID NO: 93), CRX61 (SEQ ID NO: 80), CRX23 (SEQ ID NO: 66), CRX06 (SEQ ID NO: 61), CRX62 (SEQ ID NO: 81), CRX68 (SEQ ID NO: 84), CRX35 10 (SEQ ID NO: 71), CRX04 (SEQ ID NO: 60), CRX57 (SEQ ID NO: 78), CRX56 (SEQ ID NO: 77), and GH56 (SEQ ID NO: 86); CRX61 (SEQ ID NO: 80), CRX64 (SEQ ID NO: 83), CRX04 (SEQ ID NO: 60), CRX15 (SEQ ID NO: 64), CRX06 (SEQ ID NO: 61), CRX57 (SEQ ID NO: 78), CRX56 (SEQ ID NO: 77), GH59 (SEQ ID NO: 87); and CRX33 (SEQ ID NO: 69), CRX04 (SEQ ID NO: 60), CRX06 (SEQ 15 ID NO: 61), CRX57 (SEQ ID NO: 78), DRB181 (SEQ ID NO: 257), CRX56 (SEQ ID NO: 77), GH102 (SEQ ID NO: 89), GH54 (SEQ ID NO: 85), CRX63 (SEQ ID NO: 82), CRX35 (SEQ ID NO: 71).

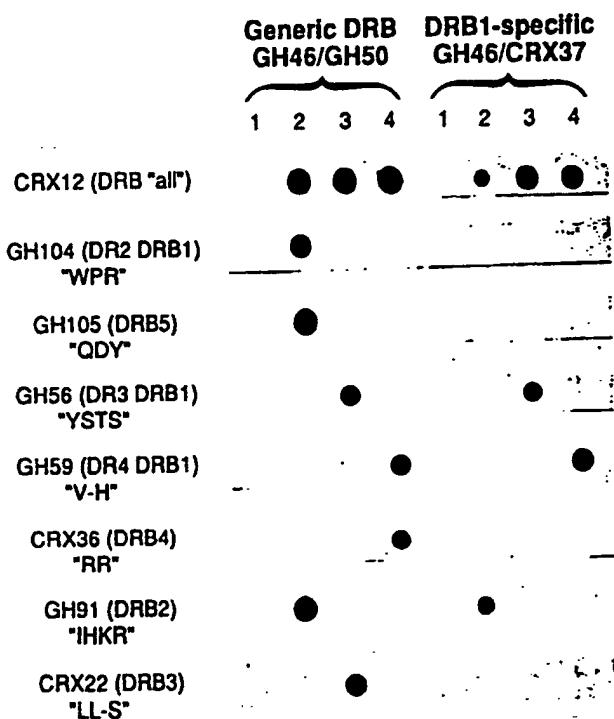
28. The method of Claim 2, wherein the primers used in step (a) are GH46 (SEQ ID NO: 67) and GH50 (SEQ ID NO: 68); the primers used in step (c) are selected from the group consisting of AB83 (SEQ ID NO: 59) and AB60 (SEQ ID NO: 57), AB82 (SEQ ID NO: 58) and AB60, AB54 (SEQ ID NO: 56) and AB60, and GH46 (SEQ ID NO: 67) and CRX37 (SEQ ID NO: 73); the first panel of oligonucleotide probes comprises two or more probes selected from the group consisting of probes CRX33 (SEQ ID NO: 69), GH104 (SEQ ID NO: 90), GH56 (SEQ ID NO: 86), GH59 (SEQ ID NO: 87), CRX49 (SEQ ID NO: 74), GH102 (SEQ ID NO: 89), GH111 (SEQ ID NO: 92), CRX34 (SEQ ID NO: 70), GH122 (SEQ ID NO: 93), CRX61 (SEQ ID NO: 80), CRX23 (SEQ ID NO: 66), CRX06 (SEQ ID NO: 61), CRX35 (SEQ ID NO: 71), CRX68 (SEQ ID NO: 84), and CRX62 (SEQ ID NO: 81); and the second panel of probes comprises two or more probes selected from a third panel of probes, wherein said third panel is selected from the group consisting of panels GH104 (SEQ ID NO: 90), DRB63 (SEQ ID NO: 140), DRB113 (SEQ ID NO: 189), CRX35 (SEQ ID NO: 71), CRX57 (SEQ ID NO: 78), CRX56 (SEQ ID NO: 77), DRB196 (SEQ ID NO: 272), DRB197 (SEQ ID NO: 273), DRB215 (SEQ ID NO: 291), and DRB216 (SEQ ID NO: 292); CRX50 (SEQ ID NO: 75), GH125 (SEQ ID NO: 94), DRB180 (SEQ ID NO: 256), GH122 (SEQ ID NO: 93), CRX61 (SEQ ID NO: 80), CRX23 (SEQ ID NO: 66), CRX06 (SEQ ID NO: 61), CRX62 (SEQ ID NO: 81), CRX68 (SEQ ID NO: 84), CRX35 (SEQ ID NO: 71), CRX04 (SEQ ID NO: 60), CRX57 (SEQ ID NO: 78), CRX56 (SEQ ID NO: 77), and GH56 (SEQ ID NO: 86); CRX61 (SEQ ID NO: 80), CRX64 (SEQ ID NO: 83), CRX04 (SEQ ID NO: 60), CRX15 (SEQ ID NO: 64), CRX06 (SEQ ID NO: 61), CRX57 (SEQ ID NO: 78), CRX56 (SEQ ID NO: 77), GH59 (SEQ ID NO: 87); and CRX33 (SEQ ID NO: 69), CRX04 (SEQ ID NO: 60), CRX06 (SEQ ID NO: 61), CRX57 (SEQ ID NO: 78), DRB181 (SEQ ID NO: 257), CRX56 (SEQ ID NO: 77), GH102 (SEQ ID NO: 89), GH54 (SEQ ID NO: 85), CRX63 (SEQ ID NO: 82), CRX35 (SEQ ID NO: 71).

29. The method of Claim 2, wherein the primers used in step (a) are GH46 (SEQ ID NO: __) and GH50 (SEQ ID NO: __); the primers used in step (c) are selected from the group consisting of AB83 (SEQ ID NO: 59) and AB60 (SEQ ID NO: 57), AB82 (SEQ ID NO: 58) and AB60, AB54 (SEQ ID NO: 56) and AB60, and GH46 (SEQ ID NO: 67) and CRX37 (SEQ ID NO: 73); the first panel of oligonucleotide probes consists of probes CRX33 (SEQ ID NO: 69), GH104 (SEQ ID NO: 90), GH56 (SEQ ID NO: 86), GH59 (SEQ ID NO: 87), CRX49 (SEQ ID NO: 74), GH102 (SEQ ID NO: 89), GH111 (SEQ ID NO: 92), CRX34 (SEQ ID NO: 70), GH122 (SEQ ID NO: 93), CRX61 (SEQ ID NO: 80), CRX23 (SEQ ID NO: 66), CRX06 (SEQ ID NO: 61), CRX35 (SEQ ID NO: 71), CRX68 (SEQ ID NO: 84), and CRX62 (SEQ ID NO: 81); and the second panel is selected from the group consisting of panels GH104 (SEQ ID NO: 90), DRB63 (SEQ ID NO: 140), DRB113 (SEQ ID NO: 189), CRX35 (SEQ ID NO: 71), CRX57 (SEQ ID NO: 78), CRX56 (SEQ ID NO: 77), DRB196 (SEQ ID NO: 272), DRB197 (SEQ ID NO: 273), DRB215 (SEQ ID NO: 291), and DRB216 (SEQ ID NO: 292); CRX50 (SEQ ID NO: 75), GH125 (SEQ ID NO: 94), DRB180 (SEQ ID NO: 256), GH122 (SEQ ID NO: 93), CRX61 (SEQ ID NO: 80), CRX23 (SEQ ID NO: 66), CRX06 (SEQ ID NO: 61), CRX62 (SEQ ID NO: 81), CRX68 (SEQ ID NO: 84), CRX35 (SEQ ID NO: 71), CRX04 (SEQ ID NO: 60), CRX57 (SEQ ID NO: 78), CRX56 (SEQ ID NO: 77), and GH56 (SEQ ID NO: 86); CRX61 (SEQ ID NO: 80), CRX64 (SEQ ID NO: 83), CRX04 (SEQ ID NO: 60), CRX15 (SEQ ID NO: 64), CRX06 (SEQ ID NO: 61), CRX57 (SEQ ID NO: 78), CRX56 (SEQ ID NO: 77), GH59 (SEQ ID NO: 87); and CRX33 (SEQ ID NO: 69), CRX04 (SEQ ID NO: 60), CRX06 (SEQ ID NO: 61), CRX57 (SEQ ID NO: 78), DRB181 (SEQ ID NO: 257), CRX56 (SEQ ID NO: 77), GH102 (SEQ ID NO: 89), GH54 (SEQ ID NO: 85), CRX63 (SEQ ID NO: 82), CRX35 (SEQ ID NO: 71).

30. A probe selected from the group consisting of RAP05 (SEQ ID NO: 312), and RAP06 (SEQ ID NO: 313).

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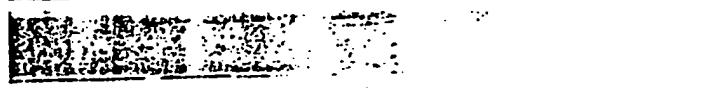
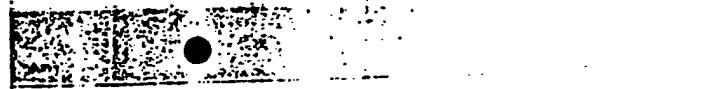
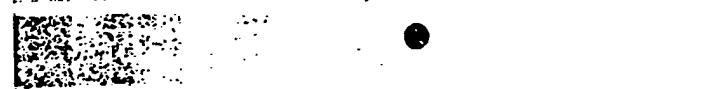
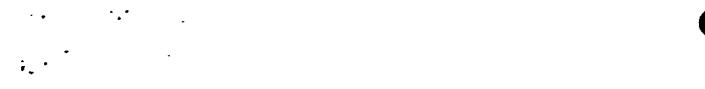
FIG. I



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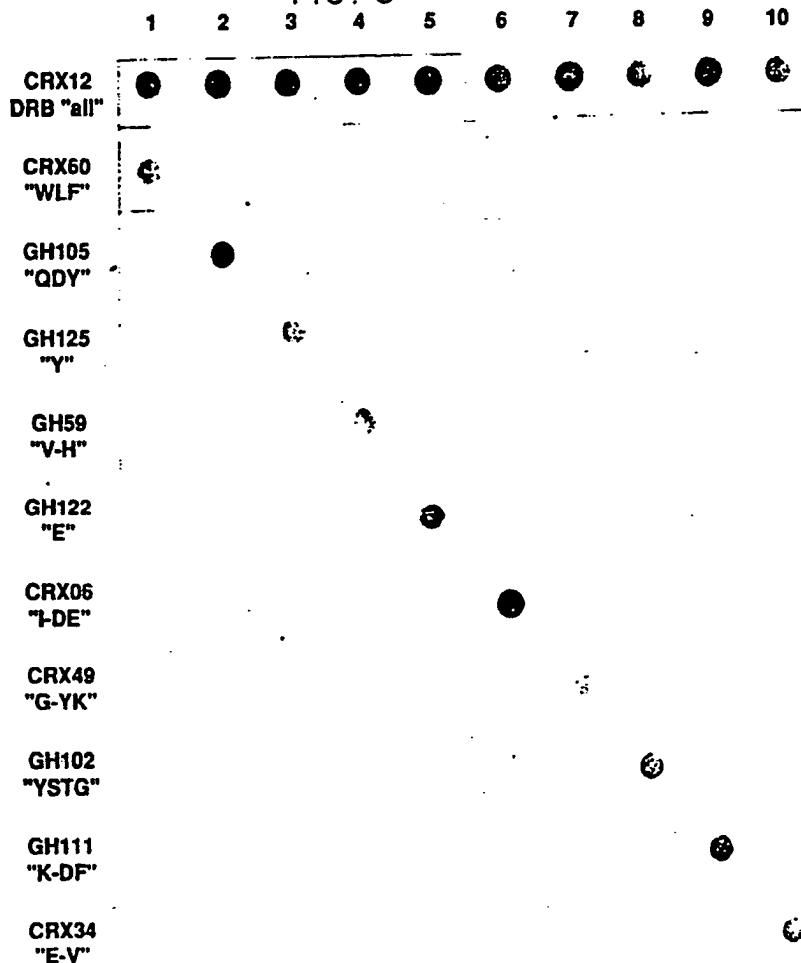
FIG. 2

1 2 3 4 5 6 7 8 9 10

CRX12
DRB "all"CRX60
"WLF"GH105
"QDY"GH125
"Y"GH59
"V-H"GH122
"E"CRX06
"I-DE"CRX49
"G-YK"GH102
"YSTG"GH111
"K-DF"CRX34
"E-V"

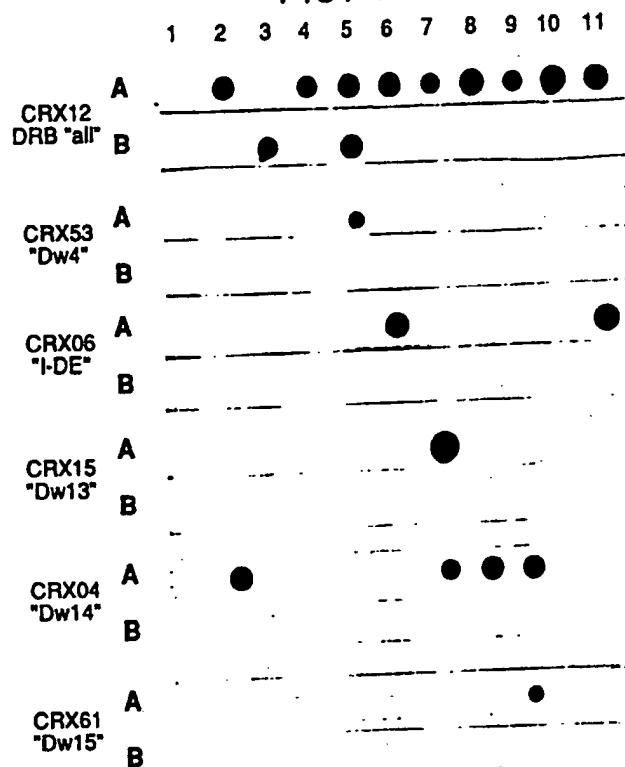
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FIG. 3



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FIG. 4



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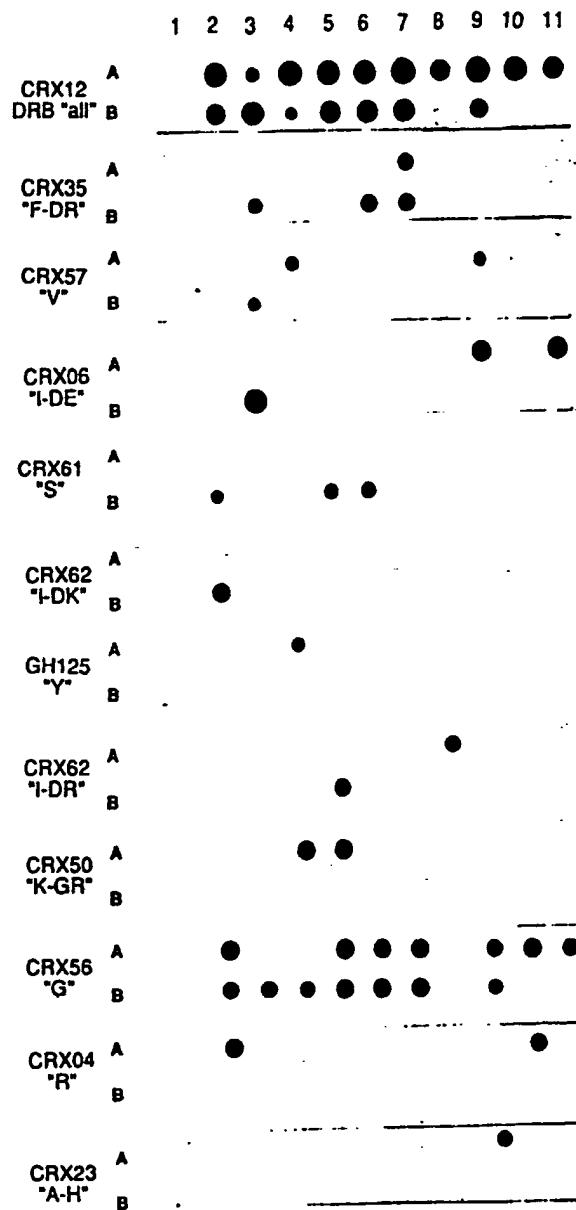


FIG. 5

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| Cell Line | GH125 "T" | CRX50 "K-GR" | CRX35 "T-DR" | CRX06 "I-DE" | CRX23 "A-H" | CRX56 "G" | CRX57 "V" | CRX04 "R" | CRX61 "S" | CRX62 "I-DK" | CRX63 "I-DR" | HLA-DRB DRB1, 0301 |
|-----------|--------------|-----------------|-----------------|-----------------|----------------|--------------|--------------|--------------|--------------|-----------------|-----------------|--------------------------|
| QBL | + | | | | | | | + | | | | 0302 |
| RSH | | + | | | | | + | | | | | 1101 |
| SPOOTIO | | | + | | | + | | | | | | 1201 |
| HERLUF | | | | | | | | | | | | |
| KOSE | | | | | + | | | | | | | |
| SLE | | | | | + | | | | | | | 1302 |
| HAG | | | | | + | | | | | | | 1303 |
| AMALA | | | | | + | | | | | | | 1402 |
| BAR P | | | | | + | | | | | | | |
| ARC | | | | | + | | | | | | | 0402/DR "PEV" 0801 |
| SPL | | | | | + | | | | | | | 0802 |
| TAB | | | | | + | | | | | | | 0803 |

FIG. 6

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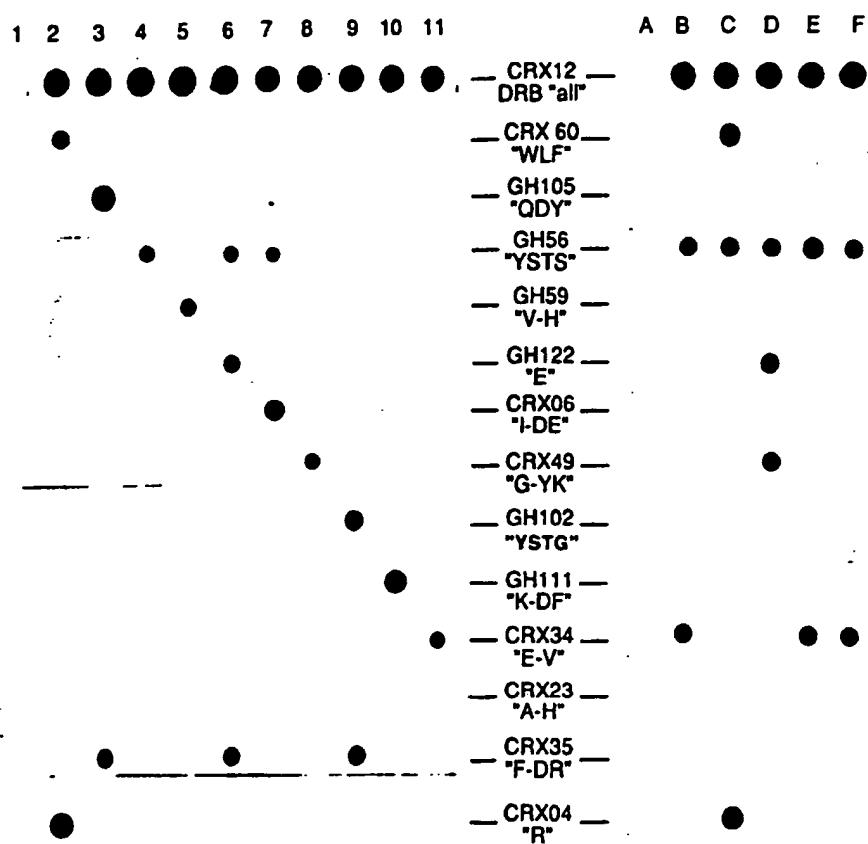
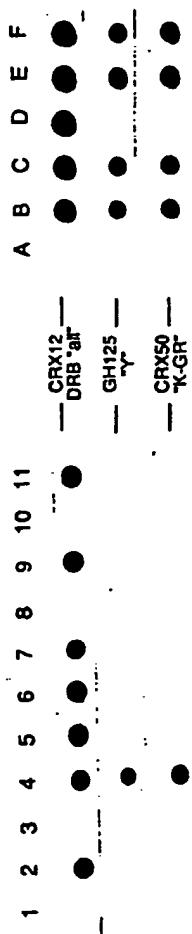


FIG. 7

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FIG. 8



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| # | CRX60 W-L-F | GH105 Q-D-Y | GH125 Y | CRX50 K-GR | GH59 V-H | GH122 E | CRX06 I-DE | CRX23 A-H | CRX09 G-YK | GH102 Y-ST6 | CRX61 K-DF | HLA-DRB DRB1* |
|------|----------------|----------------|------------|---------------|-------------|------------|---------------|--------------|---------------|----------------|---------------|------------------|
| 555 | + | | | | + | | | | + | | | 0101/0403 |
| 593 | | | | | | + | | | + | | | 0701/1301 |
| 634 | | | | | | | | | + | | | 0801/1001 |
| 766 | | | | | | | | + | | | | 0701/0701 |
| 842 | | | + | | | | | + | | | | DRW15/1101 |
| 863 | | + | | | + | | | | | | | DRW15/0301 |
| PSMS | | | | | | | + | | + | | | 0404/DRW11 |
| 2426 | | | | | ND | ND | | | | + | | 0701/0901 |
| 2446 | | | | | ND | ND | | | | + | | 0701/0701 |
| 2540 | | | | | ND | ND | | | + | | | 0101/1101 |
| 2671 | | | | | ND | ND | | | | + | | 0101/0701 |
| 2755 | | | + | | ND | ND | | | | + | | DRW15/0701 |

FIG. 9A

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| # | CRX53 K | CRX15 R-E | CRX56 6 | CRX57 V | CRX04 R | GH54 V-S | CRX62 I-DK | CRX63 I-DK | CRX34 E-V | GH56 YSTS | CRX35 F-DR | CRX12 ALL | HLA-DRB1* DRB1* |
|------|------------|--------------|------------|------------|------------|-------------|---------------|---------------|--------------|--------------|---------------|--------------|--------------------|
| 555 | + | + | | | + | | | | | + | | + | 0101/0403 |
| 593 | | | + | | | + | | | | | + | + | 0701/1301 |
| 634 | | | + | | | + | | | | + | | + | 0801/1001 |
| 766 | | | + | | | + | | | | + | | + | 0701/0701 |
| 842 | | | + | | | | | | | + | | + | DRW15/1101 |
| 863 | | | | | + | | | | | + | | + | DRW15/0501 |
| PSWS | | | | | + | | | | | + | | + | 0404/DRW11 |
| 2426 | | | | | | ND | | | | | + | + | 0701/0901 |
| 2446 | | | | | ND | | | | | | + | + | 0701/0701 |
| 2540 | | | | | ND | | | | | | + | + | 0101/1101 |
| 2671 | | | | | ND | | | | | | + | + | 0101/0701 |
| 2755 | | | | | ND | | | | | | + | + | DRW15/0701 |

FIG. 9B

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Figure 10

DRB1 Type Patterns

Probes

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Figure 11

Page 1 of 3

DRB1 Type Patterns

Prologue

DRB1 Type Patterns**Probes**

| Patterns | WLF | WPF | WAF | VWF | GTF | VTO | NDF | EVF | EAM | AAM | P-CE | + | FAM | + | W | WDF | LDF | WDF | WDF | WDF | WDF | |
|----------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|------|----|-----|----|----|-----|-----|-----|-----|-----|-----|----|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 |

DRB1*0411

Also possibly Heterozygous: DRB1 alleles 0403/0406/0407 & 0411

DRB1*1101/1104

Also possibly Heterozygous: DRB1 alleles 1101/1104 & 1305, 1101/1104 & 1403

DRB1*1102

Also possibly Heterozygous: DRB1 alleles 1102 & 1301/1302, 1102 & 1403

DRB1*1103

Also possibly Heterozygous: DRB1 alleles 1103 & 1403

DRB1*1201**DRB1*1202**

Also possibly Heterozygous: DRB1 alleles 1202 & 0802/0804

DRB1*1301/1302

Also possibly Heterozygous: DRB1 alleles 1301/1302 & 1403

DRB1*1303

Also possibly Heterozygous: DRB1 alleles 1303 & 1403

DRB1*1304

Also possibly Heterozygous: DRB1 alleles 1301/1302 & 1304, 1304 & 1403

DRB1*1305

Also possibly Heterozygous: DRB1 alleles 1305 & 1403

Page 3 of 3

DAB1 Type Patterns

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DRB1*1401 DRB1*1401 DRB1*1401

DRB1*1402 **DRB1 alleles 1402 & 1403**
Also Possibly Heterozygous: DRB1 alleles 1402 & 1403

DBE11403

00011404

גָּדוֹלָה וְתִבְרֵגָה

DRB1*0801 Also known by Heteronyms: DRB1 alleles 0801 & 08020804

טמאו טענו: 800

DBB 11.0803

DRB1-0901

DAB1•1001

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Figure 13

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 91/09294

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all)⁶

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12Q1/68

II. FIELDS SEARCHED

Minimum Documentation Searched⁷

| Classification System | Classification Symbols |
|-----------------------|------------------------|
| Int.Cl. 5 | C12Q |

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched⁸III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹

| Category ¹⁰ | Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹² | Relevant to Claim No. ¹³ |
|------------------------|--|--|
| X | WO,A,8 911 547 (CETUS CORP) 30 November 1989 see claim 1 ---- | 1,2 |
| X | IMMUNOGENETICS vol. 30, September 1989, NEW YORK US pages 208 - 213; L. FUGGER ET AL.: 'Typing for HLA-DPB1*03 and HLA-DPB1*06 using allele-specific DNA in vitro amplification and allele-specific oligonucleotide probes. Detection of "new" DPB1*06 variants' see the whole document --- | 1,2 |
| A | WO,A,8 904-875 (CETUS CORP) 1 June 1989 see page 44, line 14 - line 19 ---- | 3,6,7, 10,11, 13,24, 28,29 -/- |

⁶ Special categories of cited documents :¹⁰^{"A"} document defining the general state of the art which is not considered to be of particular relevance^{"E"} earlier document but published on or after the international filing date^{"L"} document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)^{"O"} document referring to an oral disclosure, use, exhibition or other means^{"P"} document published prior to the international filing date but later than the priority date claimed^{"T"} later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention^{"X"} document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step^{"Y"} document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.^{"A"} document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

1

07 APRIL 1992

Date of Mailing of this International Search Report

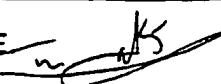
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| III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET) | | |
|--|---|--|
| Category | Citation of Document, with indication, where appropriate, of the relevant passages | Relevant to Claim No. |
| A | EP,A,0 237 362 (CETUS CORP) 16 September 1987 cited in the application see page 43, line 2 - line 6 ---- | 5,7,10, 11,13, 15,16, 18,19, 26-29 |

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO. US 9109294
SA 55499**

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| Patent document cited in search report | Publication date | Patent family member(s) | | Publication date |
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| | | JP-T- | 3504325 | 26-09-91 |
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| ----- | ----- | ----- | ----- | ----- |
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| | | AU-A- | 6996287 | 17-09-87 |
| | | EP-A- | 0459532 | 04-12-91 |
| | | EP-A- | 0459533 | 04-12-91 |
| | | JP-A- | 62214355 | 21-09-87 |
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